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Nanotechnology-Based Strategies for Rectal Anti-HIV Pre-Exposure Prophylaxis

Rute Sofia Nunes

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Nanotechnology-Based Strategies for Rectal Anti-HIV Pre-Exposure Prophylaxis

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- **Nunes R**, Araújo F, Tavares J, Sarmento B, das Neves J. Surface modification with polyethylene glycol enhances colorectal distribution and retention of nanoparticles. Eur J Pharm Biopharm. 2018;130:200-6.

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Abstract

The human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) pandemic continues to be a major global cause of illness, disability and death. Men and women engaged in unprotect receptive anal intercourse are at high risk of acquiring HIV from infected partners. The implementation of additional preventive strategies is urgent and rectal microbicides may be useful tools in reducing the sexual transmission of HIV. The design and formulation of advanced microbicides, including those based on nanotechnology approaches, may help bypassing limitations of more conventional products currently under evaluation, namely regarding the poor ability to provide and sustain protective levels of microbicide drug candidates at the colorectum. Therefore, the main aim of this work was to develop polymeric nanoparticles (NPs) as delivery systems of antiretroviral (ARV) molecules in order to be used for the development of rectal microbicides intended to prevent sexual HIV transmission.

Poly (lactic-*co*-glycolic acid) (PLGA)-based NPs were produced by nanoprecipitation and their surface modified or not with poloxamer 407, a triblock copolymer comprising polyethylene glycol (PEG) and propylene glycol (PPG) chains. Adsorption of poloxamer 407 at the surface of NPs was intended to provide a dense PEG corona that was able to confer mucus diffusive properties to otherwise mucoadhesive PLGA NPs. Both PLGA NPs and modified counterparts (PEG-PLGA NPs) featured average hydrodynamic diameter around 200-220 nm and narrow size distribution as determined by dynamic light scattering. Alongside an increase in zeta potential (ZP) to near neutral values upon modification, the presence of PEG chains at the surface of PEG-PLGA NPs was confirmed by proton nuclear magnetic resonance, energy dispersive spectroscopy and contact angle measurements. The distribution and retention patterns of fluorescently labelled PLGA NPs and PEG-PLGA NPs were further assessed in a mouse model after rectal administration by qualitative *in vivo* imaging and a quantitative method based on the fluorescence signal recovery from colorectal lavages and tissue homogenates. Both types of NPs were mainly distributed throughout the last two thirds of the colon of mice even if rapid depletion was observed. Notably, PEG-PLGA NPs showed significantly improved retention in colonic tissues and fluids at 15 min and 2 h after administration,

respectively. Also, microscopic analysis of transversal sections of the colorectum showed that PEG-PLGA NPs were able to widely distribute throughout the epithelial surface (including at tissue folds), while PLGA NPs were mainly immobilized at the central part of the gut lumen.

In order to understand the potential of developed NPs as rectal microbicide drug carriers, the ARV drug efavirenz (EFV) was associated to both PLGA NPs and PEG-PLGA NPs by adapting the nanoprecipitation procedure. The incorporation of EFV did not change substantially the colloidal properties of plain PLGA NPs and PEG-PLGA NPs. High values of association efficiency (around 85%) and drug loading (approximately 4%) were determined for both types of EFV-loaded NPs. Particles were also shown able to release EFV under sink conditions, with approximately 60% of the total drug content being release within the first hour. Association to NPs reduced the intrinsic cytotoxicity of EFV while maintaining its ARV activity, as assessed in a TZM-bl cell-based assay. Potential safety of both types of EFV-loaded NPs was also confirmed in cell-based assays using relevant epithelial cell lines of colorectal origin. Drug-loaded NPs were further shown to be readily taken up by colorectal epithelial cell lines and to mildly reduce the intestinal permeability as determined in cell monolayer models.

Finally, the local and systemic pharmacokinetic profiles of EFV-loaded PLGA NPs and EFV-loaded PEG-PLGA NPs were assessed between 15 min and 12 h after rectal administration to mice. The levels of EFV in the colorectum were generally increased when EFV was incorporated into NPs as compared to the free drug. Importantly, PEG-PLGA NPs provided higher local levels of EFV than PLGA NPs. For example, bioavailability values for EFV-loaded PEG-PLGA NPs in tissues of the middle section of the colon were around 29- and 6-fold higher than those obtained for free EFV and PLGA NPs, respectively. Systemic exposure to EFV was low in all cases. Also, both types of drug-loaded NPs were found safe upon 14 days of daily rectal administration to mice.

Overall, PLGA-based NPs were shown to be promising ARV drug carriers with potential application in the development of rectal anti-HIV microbicide products.

Keywords: efavirenz*,* mucus-penetrating particles, nanomedicine, poly(lactic-*co*-glycolic acid), pre-exposure prophylaxis, rectal administration.

Resumo[1]

A pandemia causada pelo vírus da imunodeficiência humana/síndrome da imunodeficiência adquirida (VIH/SIDA) continua a ser globalmente uma causa significativa de doença, incapacidade e morte. Homens e mulheres que praticam sexo anal receptivo desprotegido apresentam elevado risco de adquirir VIH de parceiros infectados. A implementação de estratégias de prevenção adicionais é urgente e os microbicidas rectais podem constituir instrumentos úteis na redução da transmissão sexual do VIH. O design e formulação de microbicidas avançados, incluindo aqueles baseados em nano-tecnologia, podem ajudar a ultrapassar as limitações dos produtos mais convencionais actualmente em avaliação, nomeadamente no que diz respeito à sua limitada capacidade de providenciar e manter níveis protectores de fármacos candidatos a microbicidas a nível colo-rectal. Assim, o objectivo principal deste trabalho consistiu em desenvolver nano-partículas (NPs) poliméricas como plataformas para a entrega de moléculas anti-retrovirais que possam ser utilizadas no desenvolvimento de microbicidas rectais, com o objectivo de prevenir a transmissão sexual do VIH.

NPs de ácido poli (láctico co-glicólico) (PLGA) foram produzidas por nanoprecipitação e a sua superfície foi modificada ou não com poloxamer 407, um co-polímero em tri-bloco constituído por cadeias de polietileno-glicol (PEG) e polipropileno-glicol (PPG). A adsorção do poloxamer 407 à superfície teve como objectivo proporcionar uma densa cobertura de PEG, capaz de conferir propriedades muco-difusivas às NPs de PLGA muco-adesivas. As NPs de PLGA e homólogas modificadas (PEG-PLGA NPs) apresentaram um diâmetro hidrodinâmico médio de cerca de 200-220nm e limitada distribuição de tamanho, como determinado por espalhamento dinâmico de luz. Juntamente com o aumento dos valores de potencial zeta para valores próximos da neutralidade, a presença das cadeias de PEG à superfície das NPs de PEG-PLGA foi confirmada por ressonância magnética nuclear de protões, espectroscopia de energia dispersiva e medições de ângulo de contacto. Os padrões de distribuição e retenção das NPs de PLGA e PEG-PLGA marcadas com uma sonda fluorescente foram estudados num modelo de ratinho após administração rectal, usando um modelo qualitativo de *in vivo imaging* e um método

[1] Por vontade da autora, este texto não segue as regras do Acordo Ortográfico.

quantitativo baseado no sinal de fluorescência obtido a partir dos fluídos rectais e homogeneizados de tecidos. Ambos os tipos de NPs distribuíram ao longo dos últimos dois terços do cólon de ratinho, apesar da rápida depleção observada. Notavelmente, as NPs de PEG-PLGA apresentaram uma maior retenção nos tecidos e fluídos do cólon aos 15 min e 2 h após administração, respectivamente. Além disso, a análise microscópica de secções transversais do cólon/recto evidenciou que as NPs de PEG-PLGA distribuíram-se amplamente ao longo da superfície epitelial (incluindo ao nível das pregas de tecido), enquanto as NPs de PLGA ficaram maioritariamente imobilizadas na zona central do lúmen intestinal.

De modo a perceber o potencial das NPs desenvolvidas como microbicidas rectais para entrega de fármacos, o fármaco anti-retroviral (ARV) efavirenz (EFV) foi associado às NPs de PLGA e PEG-PLGA, adaptando o procedimento de nanoprecipitação. A incorporação do fármaco não modificou substancialmente as propriedades coloidais exibidas pelas NPs de PLGA e PEG-PLGA antes da incorporação. Foram obtidos elevados valores de eficiência de associação (cerca de 85%) e capacidade de carga (aproximadamente 4%) para ambos os tipos de NPs carregadas com EFV. As partículas também foram capazes de libertar o EFV em condições *sink*, sendo libertado cerca de 60% do conteúdo total de fármaco ao longo da primeira hora. A associação às NPs reduziu a cito-toxicidade intrínseca do EFV, mantendo simultaneamente a sua actividade ARV, como confirmado em ensaios baseados em células TZM-bl. O perfil de segurança de ambos os tipos de NPs carregadas com EFV foi também confirmado em ensaios celulares usando linhas celulares epiteliais de origem colo-rectal. Mais adiante, as NPs carregadas com fármaco foram rapidamente captadas por células epiteliais colo-rectais e mostraram-se capazes de reduzir ligeiramente a permeabilidade intestinal, como estudado em modelos celulares em mono-camada.

Finalmente, o perfil farmacocinético das NPs de PLGA e PEG-PLGA carregadas com EFV foi estudado entre 15 min e 12 h após a administração rectal a ratinho. Os níveis de EFV no cólon/recto foram geralmente mais elevados quando o fármaco estava incorporado nas NPs em comparação com o fármaco livre. De notar que as NPs de PEG-PLGA proporcionaram níveis locais mais elevados de EFV do que as NPs de PLGA. Por exemplo, os valores de biodisponibilidade obtidos para as NPs de PEG-PLGA nos tecidos da secção do cólon médio foram cerca de 29 e 6 vezes mais elevados do que os obtidos para o fármaco livre e NPs de PLGA,

respectivamente. A exposição sistémica ao EFV foi baixa em todos os casos. Além disso, a administração rectal diária durante 14 dias de ambos os tipos de NPs carregadas com fármaco mostrou-se segura.

Globalmente, as NPs de PEG-PLGA mostraram-se promissoras como carregadoras de fármacos ARV e apresentam potencial para aplicação no desenvolvimento de produtos microbicidas rectais anti-VIH.

Palavras-chave: ácido poli (láctico-co-glicólico), administração rectal, efavirenz, nano-medicina, partículas penetradoras de muco, profilaxia pré-exposição.

Segue o teu destino,
Rega as tuas plantas,
Ama as tuas rosas.
O resto é a sombra
De árvores alheias.

A realidade
Sempre é mais ou menos
Do que nós queremos.
Só nós somos sempre
Iguais a nós-próprios.

(…)

Ricardo Reis

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Abbreviations and Acronyms

^{1}H NMR - Proton nuclear magnetic resonance
ACN - Acetonitrile
AE - Association efficiency
AIDS - Acquired immunodeficiency syndrome
API - Active pharmaceutical ingredient
ARV - Antiretroviral
$AUC_{0.25\text{-}12h}$ - Area under the concentration-time curve between 15 min and 12 h
C6 - Coumarin-6
CC_{50} - Half-maximal cytotoxicity concentration
CCR5 - C-C chemokine receptor type 5
CD4 - Cluster of differentiation 4
cGMP - Current good manufacturing practice
CHARM - Combination HIV Antiretroviral Rectal Microbicide
Cmax - Maximum concentration
CPP - Cell penetrating peptide
CT - Computed tomography
CXCR4 - C-X-C chemokine receptor type 4
Cy7.5 - Cyanine7.5
DAPI - 4',6-diamidino-2-phenylindole dihydrochloride
DC-SIGN - Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DL - Drug loading
DLS - Dynamic light scattering
DMEM - Dulbecco's modified Eagle's medium
DMSO - Dimethyl sulfoxide
DMSO-d6 - dimethyl sulfoxide-d6
EDS - Energy dispersive spectroscopy
EDTA - Ethylenediaminetetraacetic acid
EFV - Efavirenz
EFV-d5 - Efavirenz-d5

EMA - European Medicines Agency

ESI - Negative ionization mode

FBS - Fetal bovine serum

FDA - Food and Drug Administration

FITC - Fluorescein isothiocyanate

Frel - Relative bioavailability

FTC - Emtricitabine

GIT - Gastrointestinal tract

H&E - Hematoxylin and eosin

HBSS - Hanks' Balanced Salt Solution

HEC - hydroxyethylcellulose

HIV - Human immunodeficiency virus

HIVNET - HIV Network for Prevention Trials

HPLC-MS/MS - HPLC method coupled to tandem mass spectrometry detection

HPLC-UV - High-performance liquid chromatography with UV detection

HPV - Human papillomavirus

HSV-2 - Herpes simplex virus type 2

IBD - Inflammatory bowel disease

IC_{50} - Half-maximal inhibitory concentration

IC_{90} - 90% inhibitory concentration

IFN-γ - Interferon gamma

IL-1β - Interleukin-1β

IL-6 - Interleukin-6

IS - Internal standard

LDA - Laser Doppler anemometry

LOD - Limit of detection

LOQ - Limit of quantification

M cells - Microfold cells

MAP - mucoadhesive particles

MPP - mucus-penetrating particles

MSM - Men who have sex with men

MTT - Triazolyl blue tetrazolium bromide

MVC - Maraviroc

MW - Molecular weight

N-9 - Nonoxynol-9

NHP - Non-human primate

NIR - Near infrared

NNRTI - Non-nucleoside reverse transcriptase inhibitor

NPs - Nanoparticles

OTC - Over-the-counter

Papp - Apparent permeability coefficient

PBMCs - Peripheral blood mononuclear cells

PBS - Phosphate buffer saline

PD - Pharmacodynamics

PdI - Polydispersity index

PEG - Polyethylene glycol

PEO-PCL - Poly(ethylene oxide)-modified poly(ε-caprolactone)

PER - Permeability enhancement ratio

PK - Pharmacokinetics

PLGA - Poly (lactic-co-glycolic acid)

PPG - Polypropylene glycol

PrEP - Pre-exposure prophylaxis

RAI - receptive anal intercourse

RSI - Relative selectivity index

RTI - Reverse transcriptase inhibitor

SD - Standard deviation

SDS - Sodium dodecyl sulfate

SEM - Standard error of the mean

SERS - Surface-enhanced Raman scattering

SHIV - Simian/human immunodeficiency virus

SI - Selectivity index

SIV - Simian immunodeficiency virus

SPECT - Single photon emission computed tomography

SRM - Selected reaction monitoring

$TCID_{50}$ - half-maximal tissue culture infectious dose

TDF - Tenofovir disoproxil fumarate

TEER - Transepithelial electrical resistance

TEM - Transmission electron microscopy

tmax - Time to reach Cmax

TNBS - 2,4,6-trinitrobenzenesulfonic acid

TNF-α - Tumor necrosis factor alpha

TPGS - D-α-tocopherol PEG 1000 succinate

TVF - Tenofovir

UC - ulcerative colitis

URAI - Unprotect receptive anal intercourse

WHO - World Health Organization

ZP - Zeta potential

CHAPTER 1

Overview and Aims

1.1. Overview

The human immunodeficiency virus (HIV) infection remains a major global public health issue. Despite all efforts focused on treatment and prevention, around 36.9 million people were living with HIV/acquired immunodeficiency syndrome (AIDS) worldwide by 2017, while 1.8 million new infections were estimated to have occurred in that year alone (1). During the same period, HIV was related to roughly 1 million deaths. Sexual transmission remains by far the predominant mode of HIV transmission (2, 3). Unprotect receptive anal intercourse (URAI), both associated to heterosexual and male homosexual intercourse, is an important route for acquiring the infection and is believed to be assuming an increasing role in the pandemic (4). Importantly, the estimated per-contact probability of HIV transmission by URAI has been estimated to be around 1.4%, around 18-fold higher than the risk of acquiring the infection through unprotect vaginal intercourse (5, 6). URAI is frequently linked to men who have sex with men (MSM). However, URAI seems to be a common practice between heterosexual couples, although the prevalence has likely been underestimated (7, 8). A review study conducted by McBride & Fortenberry estimates that around 30% of heterosexual women had anal sex, at least one time (7). Several preventive measures of rectal HIV transmission are currently available, with particular focus on condom use and, more recently, oral pre-exposure prophylaxis (PrEP) with antiretroviral (ARV) drugs. Despite none being considered fully protective, current thinking in the field points towards the combination of different partially effective measures as the best approach for tackling HIV numbers (9).

In this context, rectal microbicides (topical PrEP) have been gaining substantial support as potential tools for the prevention of sexual HIV transmission. These can be defined as products containing anti-HIV compounds that are intended to be placed in the rectum in order to avoid early transmission events at the mucosal level upon sexual intercourse (10). Despite relevant progress in the field, several problems related with the formulation of products developed so far are hindering the development of an effective, safe and acceptable rectal microbicide (11). The suboptimal performance of conventional rectal dosage forms in which microbicide products are based on dictated the increased exploration of novel drug delivery

systems as platforms for the delivery of anti-HIV molecules. Substantial work from our group at i3S/INEB and others on vaginal microbicides (reviewed in (12)) support that the use of nanotechnology-based systems may also be advantageous for the development of rectal microbicides. For example, nanocarriers hold the potential to improve colorectal drug distribution and retention, penetrate mucosal tissues and enhance the interactions with HIV target cells, leading to overall favorable pharmacokinetics (PK) that may allow reducing transmission. However, very little work has been undertaken in order to effectively assess these possibilities (11, 13). One additional and important issue has to deal with the optimal properties of nanosystems for rectal delivery. In particular, the paradigm that mucoadhesive particles may be beneficial for mucosal drug delivery has been disputed, with multiple reports advocating that mucus diffusive nanosystems (also referred to as mucus penetrating) can outperform these last in terms of local distribution and even retention (14-16). Understanding how specific engineering of the mucoadhesive properties of nanocarriers intended for rectal microbicide development affects performance upon administration seems of paramount relevance.

Therefore, the present work arises from the interest in further exploring the potential of nanotechnology-based rectal microbicides and is based on the hypothesis that specifically engineered nanocarriers may contribute to the enhanced performance of candidate microbicide drugs, namely regarding the achievement of favorable PK profiles.

1.2. Aim and specific objectives

The main aim of this work is to develop nanotechnology-based carriers as delivery platforms for ARV drugs intended to be used as rectal microbicides in the prevention of sexual HIV transmission. In order to accomplish this aim, several specific objectives are proposed, namely:

- ✓ To develop and characterize relevant physicochemical and technological properties of polymeric NPs based on poly (lactic-*co*-glycolic acid) (PLGA), while considering or not the modification of their surface with a dense polyethylene glycol (PEG) corona in order to yield mucus diffusive or mucoadhesive properties, respectively;

- ✓ To test the influence of surface modification with PEG on the colorectal distribution and retention of PLGA NPs following rectal administration to an animal model;
- ✓ To associate the ARV drug efavirenz (EFV) to PLGA NPs, either modified or not with PEG, and to characterize their relevant physicochemical and technological properties;
- ✓ To ascertain on the biological properties of developed NPs, namely regarding cytotoxicity, cellular uptake, ARV activity and epithelial permeability by using relevant *in vitro* cell-based models;
- ✓ To evaluate the PK of EFV after rectal administration of drug-loaded NPs, as well as their safety, by using an animal model.

This Thesis is organized in five chapters. In *Chapter 1*, a general framework of the theme is given as well as the motivations and aim of the study. Also, the objectives to achieve the proposed aim are specified. In *Chapter 2*, a thorough review of the literature is provided concerning the overall development of rectal microbicides, including detailed background on the topic of nanotechnology-based approaches. *Chapter 3* details on the experimental work regarding the development and characterization of PLGA-based NPs, without or with surface modification by PEG, in order to obtain either mucoadhesive (PLGA NPs) or mucus diffusive (PEG-PLGA NPs) systems. Also, the role of developed NPs in the distribution and retention in the colorectum of mice after rectal administration is discussed. *Chapter 4* describes efforts undertaken in order to develop EFV-loaded PLGA NPs and EFV-loaded PEG-PLGA NPs and characterize their physicochemical and technological properties. Studies on the interactions between nanosystems and cells are also detailed, namely regarding toxicity, ARV activity, cell uptake, and drug epithelial permeability and retention. Additionally, this chapter presents and discusses data regarding the ability of developed drug-loaded NPs to modulate the PK of EFV after rectal administration to a mouse model. Data concerning the safety of NPs after 14 days of once daily rectal administration is further described. Finally, *Chapter 5* includes the overall conclusions of the work as discusses several possibilities regarding future studies.

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CHAPTER 2

Literature Review

The information provided in this chapter was based in the following publications:

Melo M*, **Nunes R***, das Neves J, Sarmento B. Rectal administration of nanosystems: from drug delivery to diagnostics. *Submitted for publication*.

Nunes R, Sarmento B, das Neves J. Formulation and delivery of anti-HIV rectal microbicides: Advances and challenges. J Control Release. 2014;194c:278-94.

2.1. The rectum: basic aspects

2.1.1. Biology of the rectum

The rectum comprises the distal 15-20 cm of the gastrointestinal tract (GIT), with a total surface area of 200-400 cm^2 (1, 2). It extends from the sigmoid colon to the pectinate line, where the anal canal begins (**Figure 2.1**). It can be divided into three distinct parts: the upper, middle and lower rectum (3). The mucosa of the rectum is composed of simple columnar epithelium forming crypts of Lieberkühn (or intestinal glands) and presenting goblet cells, which rests over a lamina propria bearing multiple lymphoid cells and individual lymph nodes (4). The lack of villi and microvilli provides a relatively small surface area for absorption as compared to the small intestine. Also, Paneth cells, the main producers of antimicrobial factors in the small intestine, are usually absent in healthy colon and rectum (5). Proximal to the pectinate line the simple columnar epithelium makes an abrupt transition to non-keratinized stratified squamous epithelium (anorectal junction), which extents continuously towards the keratinized stratified squamous epithelium of the skin at the external anal sphincter (6).

Blood supply and return are assured by rectal arteries and rectal veins, respectively (7). The superior rectal arteries irrigate the upper and middle rectum, while the middle rectal artery irrigates the lower rectum and proximal anal canal. The inferior rectal arteries supply the anus and the anal sphincters. The venous drainage of the rectum and anus correlates to and travels with its arterial supply. The superior rectal vein drains the upper and middle rectum into to the portal vein. The venous blood of the lower rectum and anal canal is collected by the middle and inferior rectal veins and drains into the inferior vena cava. Thus, drugs absorbed at the lower rectum avoid, at least partially, the hepatic first-pass metabolism, reaching directly the systemic circulation. In contrast, drugs absorbed at the upper rectum undergo pronounced hepatic first-pass effect. Nevertheless, this relation is not straightforward as extensive anastomoses connect all three rectal veins throughout the rectum (7).

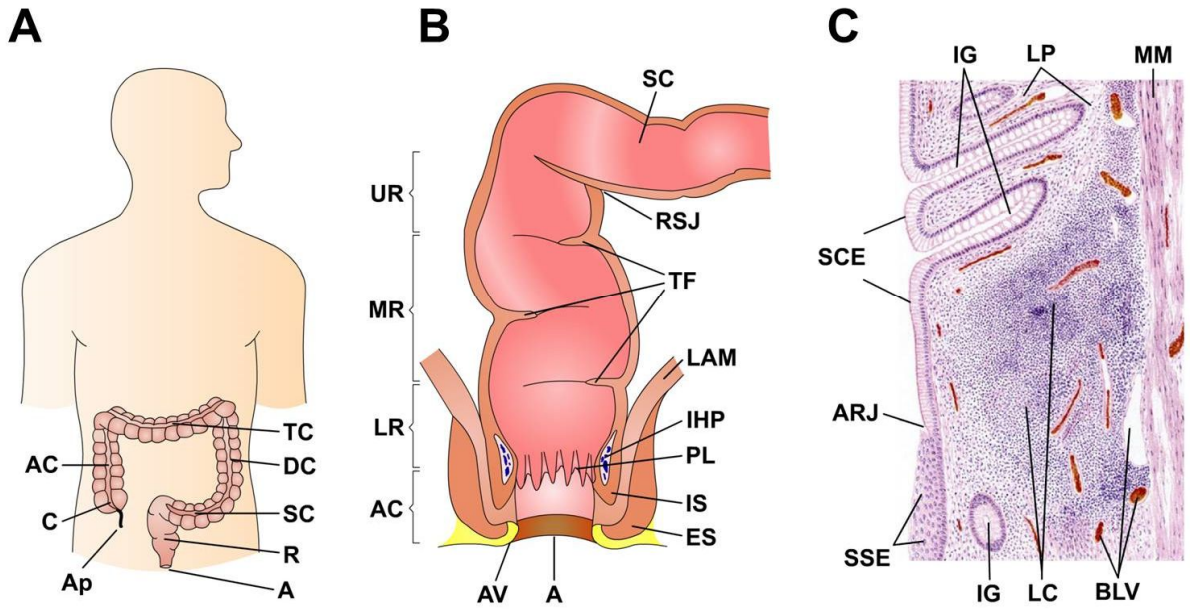

Figure 2.1. Schematic representation of the anatomy and histology of the human rectum: (**A**) gross anatomy and position of the rectum in relation to the colon and anus, (**B**) detailed longitudinal anatomy of the colorectum and anus, and (**C**) histology of the anorectal mucosa (longitudinal section, H&E stain, low magnification). Image in (C) reproduced from reference (4), Copyright (1999), with kind permission from Springer Science and Business Media. Legend: A, anus; Ac, anal canal; AC, ascending colon; Ap, appendix; ARJ, anorectal junction; AV, anal verge; BLV, blood and lymphatic vessels; C, cecum; DC, descending colon; ES, external sphincter; IG, intestinal glands (crypts of Lieberkühn); IHP, internal hemorroidal plexus; IS, internal sphincter; LAM, levator ani muscle; LC, lymphoid cells; LP, lamina propria; LR, lower rectum; MM, muscularis mucosa; MR, middle rectum; PL, pectinate line; R, rectum; RSJ, rectosigmoid junction; SC, sigmoid colon; SCE, simple columnar epithelium; SSE, stratified squamous epithelium; TC, transverse colon; TF, transverse folds; UR, upper rectum.

Broad networks originating in the mucosa and submucosa give rise to lymphatic vessel. The lymph of the descending colon, sigmoid colon and upper rectum are collected by inferior mesenteric lymph nodes and drains into the left lumbar trunk. The lymph of the middle and lower rectum drains to internal and external iliac lymph nodes, which then converge to the lumbar trunk (8). The contribution of the lymphatic vessels for drug absorption is not well established since lymph flow is residual as

compared to blood circulation. However, lymphatic drainage may also allow avoiding the hepatic first-pass effect.

The mucosal lining of the GIT is covered by a mucus layer which provides protection against mechanical stress (*e.g.*, during defecation) and is an efficient barrier for xenobiotics and pathogens (9). Rectal mucus is mostly produced by goblet cells and composed mainly of water and mucins (<5%), although presenting minor amounts of other components, namely antimicrobial factors like lysozymes, defensins, cathericidins and secretory phospholipase A2 (10, 11), some of which have been shown to inhibit HIV (12). The consistency of mucus is largely influenced by its content in mucins, which gradually increases along the colon and towards the rectum (13). The rectal fluid layer is around 150 μm in thickness (range 75-250 μm) (14), with an estimated turnover time of 3-4h as determined *ex vivo* using rectal biopsies (15). Mucus presents a slightly alkaline pH, around 7-8, with minimal buffering capacity (16). The total amount of fluid at the rectal cavity is estimated at 1-3 mL (17).

Another important issue to be considered is microbiota at the colorectal environment. The amount of microorganisms increases along the GIT and towards the colorectum, being mainly composed by anaerobic Gram positive and Gram negative bacteria, and constituting an important natural mechanism of defense against pathogens (18). However, the role of microbiota on the prevention of viral infections is poorly understood and some evidence points out that it can either protect or promote viral transmission and/or spread, through direct or indirect mechanisms (19). For example, the role of bacterial lipopolysaccharide in HIV replication or inactivation has been the focus of different studies, however, with inconclusive results (20, 21). Also of interest, rectal microbiota produce hydrolytic and reductive enzymes which can influence the metabolism of drugs (22).

2.1.2. Rectal drug delivery

The administration of drugs into the rectum can provide a suitable way to produce either local or systemic effects, and constitutes mainly an alternative to other mainstream delivery routes, namely the oral one. A synopsis of the main advantages and limitations of the rectal route is presented in **Table 2.1**. The possibility of rectal delivery for different drugs (*e.g.*, anticonvulsants, analgesics, anti-inflammatories, anti-asthmatics, antiemetics, antibiotics) has been studied and, in many cases, their use is well established in clinical practice. In particular, rectal delivery may be useful for managing colonic disease due to the short anatomical distance and continuum between these two sites of the GIT. Also, the systemic delivery of peptides and proteins through the rectal route has been under investigation but often with moderate success and requiring substantial formulation efforts (23, 24). The rectal mucosa may also constitute a potential site for vaccine administration in order to obtain topical and systemic immune responses against various microorganisms, including HIV (25).

Table 2.1. Advantageous and disadvantageous features of the rectal drug delivery route.

Advantages	Disadvantages
Non-invasive	Erratic drug absorption
Relatively stable environmental conditions [(a)]	Absorption influenced by local pathologies
Partial avoidance of hepatic first-pass effect	Presence and traffic of stool
Low enzymatic activity [(b)]	Acceptability (cultural) issues
Relatively high amounts of drug can be administered	May cause irritation [(c)]
May limit systemic exposure/adverse effects [(c)]	May cause discomfort
Rapid onset of pharmacological effect	Defecation urge
Allows self-administration	Leakage
May be used in unconscious subjects	
Not limited by emesis	
Suitable for pediatric and geriatric patients	
Painless	

[(a)] pH, amount of fluid, among others; [(b)] as compared to the upper GIT; [(c)] dependent on drug and drug product characteristics.

The relatively smaller surface area of the rectal mucosa, as compared to the small intestine, limits its absorptive capacity. Even so, rich blood and lymphatic drainage may allow obtaining significant systemic levels for different drugs. Some compounds can even have higher bioavailability upon rectal delivery as compared to the oral route due to the partial avoidance of the hepatic first-pass effect (26, 27). Drugs administered in the rectum require to be released from dosage forms and dissolve in local fluids in order to be absorbed (1). This process will influence the extension and rate of absorption and is dependent on multiple factors, namely: (i) local environment and health state (temperature, fluids characteristics – pH, volume, surface tension, viscosity, buffer capacity, thickness –, microbiota and host mucosal metabolism, presence of feces), (ii) host behavior and health state (defecation, involuntary expulsion, acceptability, constipation, local trauma, luminal pressure or periodic contractile activity of the rectal wall), (iii) drug properties (solubility, partition coefficient, particle size and surface, molecular weight – MW –, ionization degree, concentration), and (iv) dosage form/delivery system features (composition, melting point/dissolution ability of solid systems, surface tension, dielectric properties, rheological properties) (1, 2, 28). Once dissolved in the luminal fluid, drugs need to cross different barriers in order to reach the blood and/or lymph, such as the mucus layer, apical and basal cell membranes, tight junctions, basement membranes, intracellular compartments and walls of lymph vessels or blood capillaries (29). Rectal drug absorption occurs mainly by passive transport, and active molecules may be absorbed by the transcellular or paracellular pathways, depending on their physicochemical features (30). The transport by the first pathway is mainly for drugs with some degree of lipophilicity, and is well explained by the pH-partition hypothesis. According to this last, only non-ionized molecules of a compound will be available for diffusion across membrane lipids. The second is a common pathway for many water-soluble and poorly lipid-soluble molecules as well as ionized drugs (30).

Absorption can be a double edge-sword depending on the final intent of the administered drug (*i.e.*, local or systemic action). In many ways, absorption depends on dosage forms/delivery systems used and can be modulated by changing the pharmaceutical formulation, namely by using absorption enhancers or enzymatic inhibitors (31). Drugs are usually administered in the rectum as liquid (solutions, suspensions, emulsions) – commonly referred to as enemas or microenemas

depending on volume –, semisolid (gels, creams, ointments) and solid (suppositories, capsules) dosage forms (32, 33). With the exception of solid ones, other dosage forms require the use of an applicator. Suppositories constitute most of current products administered rectally in clinical practice. The base composition of commercialized rectal products varies widely, conferring different properties that can change the degree and rate of drug release and absorption. So, the physicochemical characteristics of the drug and its desirable release pattern should be taken into account when excipients/dosage forms are chosen. For instances, the use of liquid dosage forms allows faster absorption since drug release and dissolution issues are generally circumvented (1). Drug absorption is slower when solid systems such as suppositories are used, although this is tremendously dependent on composition (28). The spreading features of a product are highly dependent on properties such as viscosity and mucoadhesion, and may determine the drug fraction bypassing hepatic first-pass metabolism and influence the effectiveness of drugs administered for local action. Overall, drugs administered as suppositories are confined to the rectum and sigmoid colon while enemas, depending on their volume, can reach more proximal portions of the colon. Foams seem to have an intermediary behavior between enemas and suppositories. Further, smaller volumes are linked with greater rectal drug retention, while volumes higher than 80 mL usually stimulate defecation (1).

2.2. Nanotechnology-based approaches for rectal drug delivery

2.2.1. Role of nanotechnology in rectal drug delivery

Nanotechnology is often seen with overwhelming optimism when considering healthcare applications, despite many well-known limitations and challenges (34). Beyond the common hipness and popularity associated with nanomedicine, there are actually several possible advantages of using nanocarriers that could be interesting for rectal administration of active compounds. Alongside data obtained from studies specifically addressing rectal administration of nanosystems, a set of possibilities can also be inferred from studies involving other drug delivery routes, namely the oral one. From a general perspective of pharmaceutical formulation, nanotechnology-based approaches have been successful in solving poor drug solubility, which is known to undermine the use of various promising compounds (35). In the particular case of the rectal compartment, the low amount of fluids that are naturally present or that can be administered makes solubility a main topic to be considered. Relatively simple solutions such as nanosizing insoluble drugs may be useful. As an example, Rachmawati *et al.* (36) observed enhanced anti-inflammatory effects for D-α-tocopherol PEG 1000 succinate (TPGS)-stabilized curcumin nanosuspension (mean particle diameter of ≈210 nm) after multiple rectal administrations to a trinitrobenzene sulfonic acid (TNBS)-induced colitis rat model, as compared to a curcumin suspension (>7 μm and also containing TPGS). The use of drug nanocarriers can further be helpful in allowing the production of physicochemical stable systems containing labile compounds such as nucleic acids or proteins. Hard to formulate drugs may found in nanotechnology a suitable way to abbreviate degradation during storage and even upon administration in the lower GIT (37). Control of drug release is also possible with nanocarriers (38), even though other type of larger systems may be better suited for such purpose.

The distribution and retention of drugs upon rectal administration are important factors in defining drug efficacy. Depending on specific colloidal properties, nanocarriers can help enhancing drug transport along and across mucus and coverage of the mucosal surface, as well as increase residence. This particular topic

has seen important advances over recent years and is discussed in more detail over the next section. Nanosystems present the additional potential for targeted drug delivery along the colorectum, particularly when diseased tissues can be distinguished from healthy counterparts (*e.g.*, in ulcerative colitis (UC) or cancer) (39-41). In these cases, distinct pathophysiological features of the mucosal wall can be used for engineering targeted nanosystems. For example, Lamprecht *et al.* (42) reported on the accumulation of negatively-charged polystyrene particles at inflamed tissue areas of the colon following deep intrarectal administration to rats presenting TNBS-induced colitis. Such effect could be moderately enhanced by simply reducing the size of the particles from ≈1 μm or above to ≈100 nm. A clear mechanistic explanation for these observations was not provided but differences in bioadhesion as related to different properties of local mucus (thicker but possessing looser structure in colitis) and tissue charge (more positive in colitis (43)), as well as disruption of the epithelial barrier, may be involved. Indeed, differences between differently sized negatively-charged particles were not seen when considering normal colon mucosae, including in healthy rats (42). The presence of an abnormal number of immune cells in inflammatory conditions of the colon may also have contributed to the enhanced uptake of NPs and, thus, tentatively lead to increased drug concentrations at damaged tissues (44). Importantly, a first in-human trial testing either PLGA-based NPs (≈250 nm) or microparticles (≈3 μm) failed to essentially replicate previous animal data (45). Particles were administered intrarectally in a normal saline enema containing 10% albumin, which was retained for 2 h *in loco* before expulsion, and further probed by confocal laser endomicroscopy. While larger particles, but not NPs, were shown to mildly target ulcerated tissue in inflammatory bowel disease (IBD) patients, accumulation was overall much lower. Although a more objective explanation for these discrepancies seems elusive, interspecies variability and substantial differences in the colloidal properties of particles (polymer type, size, surface charge, albumin coating) may be accountable. Again, proper engineering of nanosystems appears to be of paramount importance for overall performance.

Targeting specific biological moieties that are naturally present at the epithelium by using functionalized nanocarriers may further be useful as a strategy for enhancing drug delivery, namely for cell targeting (46). Another interesting feature of nanosystems has to deal with the possibility of modulating drug permeability at the

colon and, thus, influence bioavailability. Nanoparticulate systems are able to actually penetrate colorectal tissue, which could be beneficial for allowing the establishment of a drug depot that sustains local drug residence, as well as for targeting structures or cells present at the lamina propria (47). Data from experiments using *ex vivo* human rectal mucosa biopsies obtained from healthy individuals and IBD patients indicate that translocation of negatively-charged PLGA-based NPs (≈250 nm) across intact epithelium is limited but increases in damaged mucosa (45). Limited evidence further suggests that at least some type of nanosystems, namely liposomes of ≈300 nm in diameter, may partially reach systemic circulation upon rectal delivery and distribute throughout various distant tissues (48). However, recent work using PEG-modified silica-based nanocapsules (≈120 nm) indicates that such absorption and long distance recovery of NPs is typically scanty following intrarectal administration (49). Lack of systemic exposure to nanosystems may be considered of interest as it may favors localized pharmacological action and reduced potential toxicity.

Lastly, a note is due to nanosystems possessing intrinsic pharmacological activity rather than acting as drug carriers. Dendrimers, in particular, have been comprehensively tested for their potential use as rectal microbicides in order to prevent infection by multiple pathogens, including HIV (50-52). The colloidal nature of these structures allows enhancing exposure of surface chemical groups responsible for interactions with pathogen or host molecular targets involved in transmission. Silver NPs (53, 54) and glass beads coated with nanolayers of silver (55) have also been studied for the treatment of colitis due to their intrinsic anti-inflammatory activity.

2.2.2. Distribution and retention of nanosystems following rectal administration

One of the potential advantages of using nanosystems has to deal with their ability to modulate self-distribution, as well as residence, along the terminal GIT. Transport in the colorectal lumen and beyond is conditioned by several factors related not only with the mucosal environment but also with the intrinsic characteristics of nanosystems such as size and surface chemistry. Indeed, as discussed in the following, suitable engineering of the colloidal features of

nanosystems plays a key role in modulating biodistribution upon rectal administration.

As in the case of other type of materials, nanosystems typically undergo rapid depletion towards the exterior following administration in the rectum as a result of the natural bowel movement and defecation reflex. The presence of stool or practice of anal sex may additionally influence clearance. Moreover, retrograde transport is thought to be naturally limited, although different studies demonstrated that nanosystems can indeed reach far into the upper parts of the colon and feature more or less extended residence. For example, Zavaleta *et al.* (49) showed that surface-enhanced Raman scattering (SERS) NPs (≈120 nm) – comprising gold core/silica shelled capsules modified at the surface with PEG (2-5 kDa) and ^{64}Cu – distributed throughout the colon following rectal administration to mice, as assessed by Raman imaging and micropositron emission tomography. Although the depth of catheter insertion into the colon was not disclosed (but likely several centimeters) nor quantitative colon spreading studies were performed, data suggest that NPs could be transported retrogradely, at least in some animals (**Figure 2.2**) (49). The use of a relatively large volume (200 μL) may aided spreading. Also, distribution into the blood and other organs (including the spleen and liver) was shown negligible. Another report by the same group confirmed these data and further indicated that NPs were rapidly washed out from the colon following a single rectal administration, without apparent signs of systemic toxicity (56).

One particularly limiting physiological factor for colorectal distribution and retention is the presence of mucus, a viscoelastic gel composed of water and entangled mucin fibers, which constitutes a stringent barrier for particle diffusion (57, 58). The ability of nanosystems to bond mucins determines their adhesive behavior and, thus, overall mobility. The use of mucoadhesive nanosystems has been conventionally considered beneficial for enhancing drug retention at mucosal sites and a popular approach to improve either local or systemic drug bioavailability (59). Strategies involving the modification of the surface of nanosystems in order to yield enhanced molecular entanglement and covalent or non-covalent bonding with mucin fibers are recognized for increasing mucoadhesiveness (60). However, strong adhesion of nanosystems to mucins impairs effective transport across mucus thus limiting distribution not only towards the underlying mucosal tissue but also

retrogradely into the colon. Also, mucoadhesive systems are mostly immobilized at the outer, non-adherent layer of mucus, which undergoes continuous and rapid clearance (57, 61).

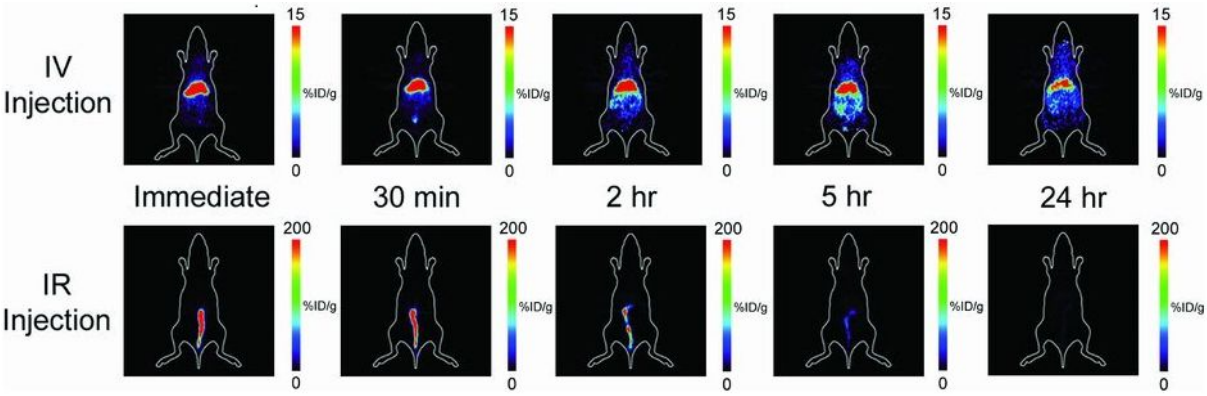

Figure 2.2. Micropositron emission tomography images of the accumulation of 64Cu-modified SERS nanoparticles post intravenous (IV) injection (top panel) versus post intrarectal (IR) injection (bottom panel). The images represent a coronal slice of a single mouse taken at various time points; immediately, 30 min, 2 h, 5 h, and 24 h after either IV or IR injection. Notice the significant difference in the accumulation of ^{64}Cu-modified SERS nanoparticles in mice receiving an IV injection, where uptake is localized to the liver, versus mice receiving an IR injection, where uptake is localized to the colon. Colored scale bar to the right of each image represents ^{64}Cu-SERS uptake where red represents most uptake and black represents no uptake in units of percentage of injected dose per gram (%ID g^{-1}). Reprinted with permission from (62). Copyright (2011) Wiley-VCH Verlag.

Conversely, mucus-diffusive (or mucus-penetrating) nanosystems can migrate to the inner layer of mucus, which is less prone to luminal shearing, and even have easier access to underlying tissues. Therefore, the use of non-mucoadhesive nanosystems has been gaining advocates over recent years as an interesting approach to promote not only spreading along mucosal sites but also enhance overall residence (63, 64). The most successful strategy for producing mucus-diffusive nanosystems has been developed by Hanes and collaborators (65-67) and involves dense surface modification of nanosystems with PEG featuring adequate

molecular mass for preventing interactions with mucin, alongside adequate particle size control down to dimensions that allow fitting the mucus mesh spaces. Still, other approaches have also been recently proposed including surface modification of nanosystems with alternative non-mucoadhesive polymers or association with mucolytic agents (68-71). In the specific case of rectal delivery applications, Maisel *et al.* (72) modified polystyrene NPs of different sizes with PEG (5 kDa) and found that this approach led to an overall increase in migration of nanosystems across mucus, as well as to an extensive coverage of the epithelial surface after administration to mice (**Figure 2.3**). Contrariwise, mucoadhesive counterparts (i.e., without PEG modification) distributed poorly and largely aggregated at the intestinal lumen. Size of NPs also played an important role: 40-100 nm PEG-modified NPs featured a more uniform distribution along the epithelial surface as compared to larger ones (200-500 nm). This fact may well be associated to the available space within the colorectal mucus mesh, which sterically hinders transport of larger nanosystems. Another important finding of this study relates with different patterns of NPs transport due to pathological changes. For example, colorectal distribution of 200 nm mucus-diffusive NPs was enhanced in a TNBS-induced colitis mouse model, which feature a thicker yet looser mucus barrier, as compared to healthy animals. Moreover, mucus-diffusive NPs appeared to accumulate better than mucoadhesive counterparts in ulcerative lesions (72). The use of a hypotonic vehicle was further shown beneficial for administering mucus-diffusive NPs due to the promotion of convective transport (72, 73). In a subsequent study by the same research group, similar behavior was also described for NPs modified with PEG of higher molecular mass (40 kDa) after intrarectal administration to mice (74). Despite these interesting findings, actual time-dependent, quantitative retrograde migration and overall colon retention of PEG-modified NPs were not determined. Assessment of such data is paramount for fully understanding the potential of mucus-diffusive NPs as carriers for rectal drug delivery and, thus, additional investigation is required.

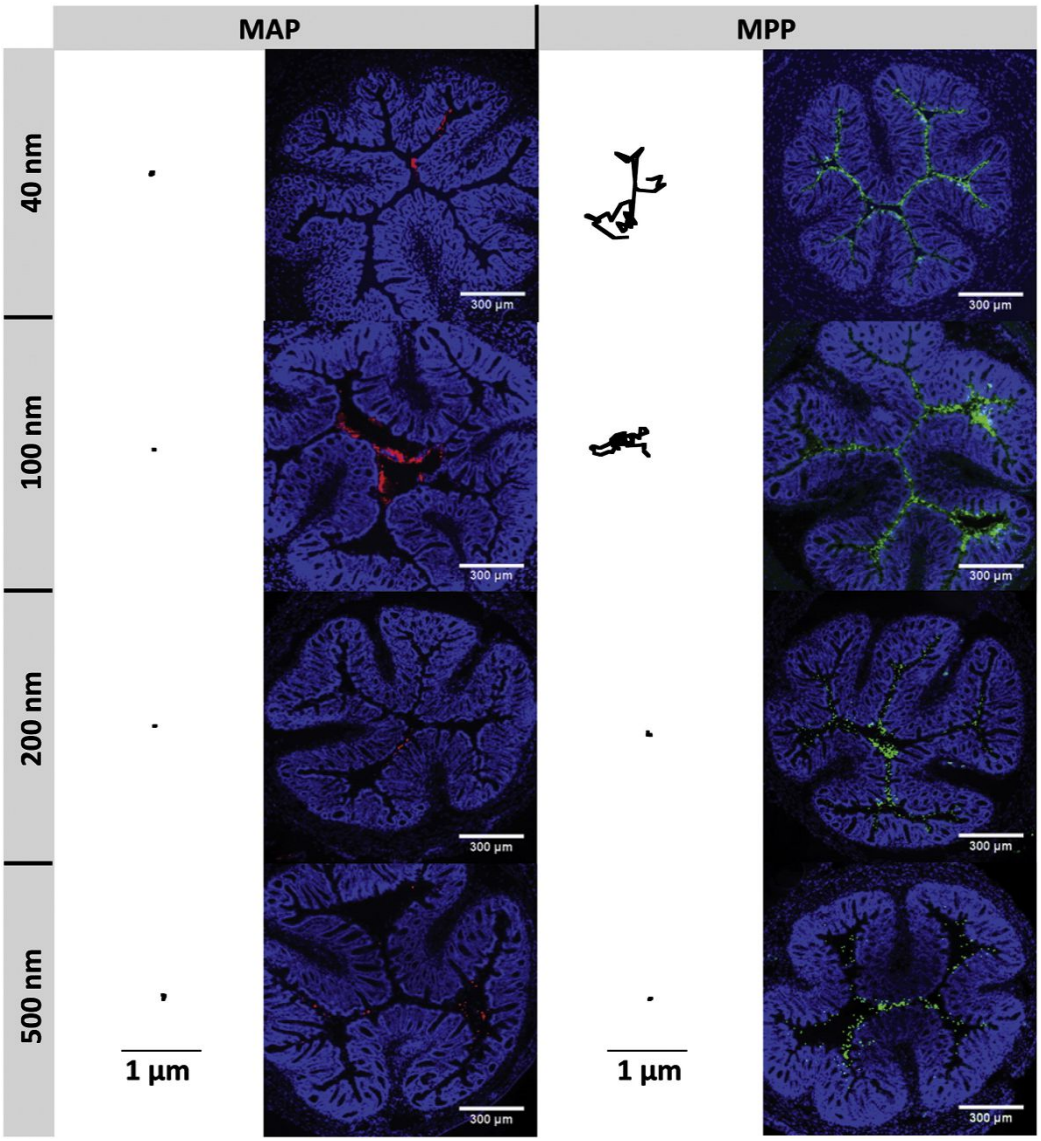

Figure 2.3. Trajectories in colorectal mucus and distribution of mucoadhesive particles (MAP) and mucus-penetrating particles (MPP) in the mouse colorectum. Trajectories representative of three seconds of movement for 40, 100, 200, and 500 nm MAP and MPP in mucus on freshly excised mouse colorectal tissue as assessed by multiple particle tracking video microscopy. Black scale bars indicate 1 μm for all trajectories. Distribution in transverse colonic cryosections after rectal administration of 40, 100, 200, and 500 nm MAP (red) or MPP (green). Cell nuclei are stained with DAPI. White scale bars indicate 300 μm for all distribution images. Images are representative of $n \geq 3$ mice. Reprinted from (72), Copyright (2014), with permission from Elsevier.

2.3. Rectal transmission of HIV

Understanding the pathogenesis of HIV transmission through mucosal surfaces, namely the rectal mucosa, is crucial for the development of rectal microbicides. Despite all efforts undertaken since the discovery of the virus, there are still some loose ends regarding the understanding of the complete mechanism of rectal infection. Transmission is mediated by HIV-target cells (*i.e.*, T cells, macrophages and dendritic cells) present at the lamina propria of the mucosa after rectal deposition of semen containing cell-free or cell-associated virions (**Figure 2.4**) (75). *In vivo* evidence seems to point towards cell-free virus as being the most important source for infection (76), even if recent animal studies showed that cell-associated virus (simian immunodeficiency virus – SIV) may be more efficiently transmitted (77, 78). Once in the rectum compartment, HIV appears to take advantage of multiple mechanisms to overcome the epithelial barrier and achieve target cells (**Figure 2.4, A-E**). For example, the presence of micro ulcerations or mechanical breaching, frequently resulting from sexual intercourse, provides a direct access to the lamina propria. The occurrence of concomitant infections, namely by herpes simplex virus type 2 (HSV-2) (79) or human papillomavirus (HPV) (80), or the presence of HIV virions (81) can also induce epithelial damaging and target-cell recruitment. The virus can even cross intact rectal epithelium by transcytosis through epithelial cells (82) or be taken up by microfold cells (M cells) overlaying lymphoid follicles (83). Also, transmission can occur via dendritic cells as mediated by a trans-infection mechanism involving dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) (84).

Overall, only a very small number of events are required for the virus to reach target cells at the lamina propria. These cells typically express the cluster of differentiation 4 (CD4) receptor, which render them susceptible to viral infection, and at least one of two co-receptors, C-C chemokine receptor type 5 (CCR5) and C-X-C chemokine receptor type 4 (CXCR4), even if CCR5-tropic (R5) viruses are more efficiently transmitted and account for most of the HIV primary infections worldwide (85). After the establishment of a founder population of HIV-infected cells and its mucosal expansion, viral dissemination to nearby lymph nodes and then to

secondary lymphatic tissues occurs, with consequent establishment of irreversible systemic infection (86).

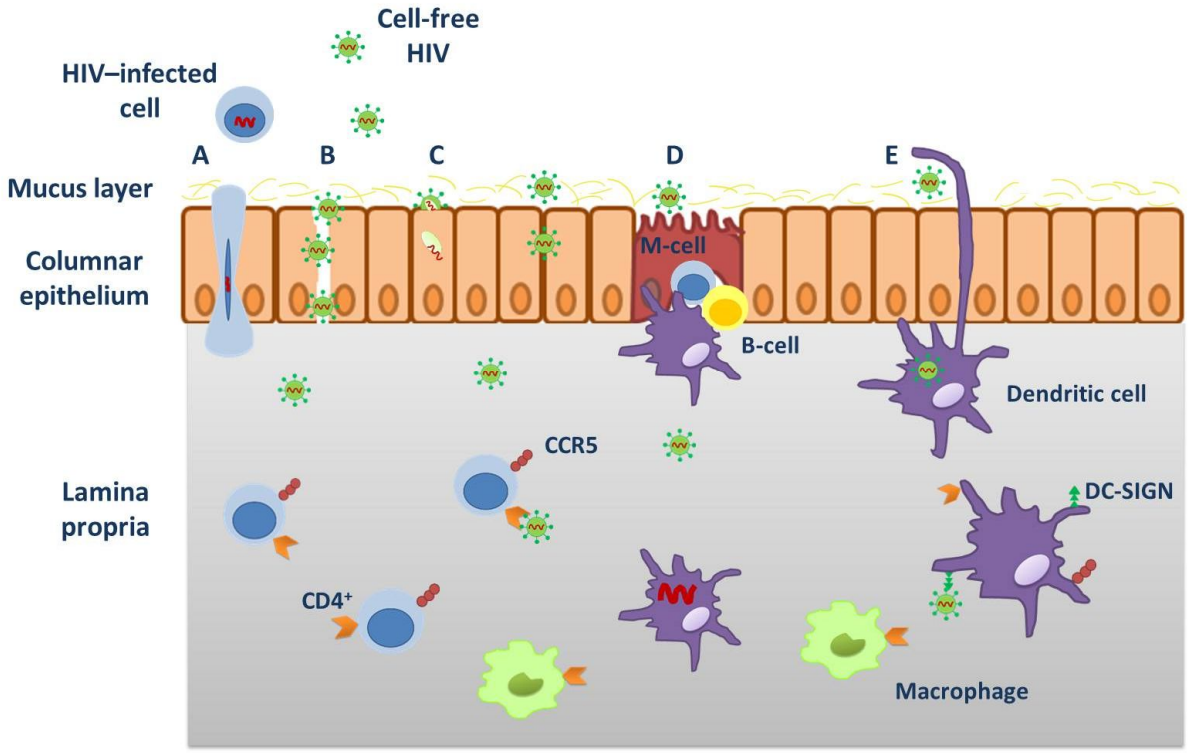

Figure 2.4. Schematic representation of the possible mechanisms for HIV transmission across the colorectal mucosa. The virus can access the underlying lamina propria by (**A**) epithelial transmigration of HIV-infected cells, (**B**) direct access of the free virus through physical breaches in the epithelium, (**C**) transcytosis of HIV across epithelial cells, (**D**) transcytosis of the free virus across specialized microfold cells (M-cells), or (**E**) binding of HIV to dendritic cells (DC) followed by non-endocytic transfer. Once at the lamina propria, the virus is presented to target cells and undergoes local amplification before systemic dissemination of the infection.

Notably, increased risk of HIV acquisition through URAI as compared to the vaginal route can be explained, at least partially, by anatomical, physiological, immunological and histological features (87). For example, the near neutral pH and

minimal buffer capacity of rectal fluids provide no to little protection against infection, contrasting with the acidic pH of the vagina conferred mostly by microbiota-produced lactic acid, which is able to protect against sexually transmitted pathogens including HIV (88). The simple columnar epithelium of the colorectal mucosa turns this natural barrier more vulnerable to physical damaging during sex, thus increasing the likelihood of the virus to reach target cells (89). Contrasting with the vaginal mucosa, lymphocytes in colorectal tissues are constitutively activated, thus increasing viral susceptibility (90). Furthermore, the open ended structure of the rectum provides a greater surface area for infection as compared with the vaginal cavity. Some studies have been conducted to establish the potential distribution of semen/HIV particle surrogates in the GIT following rectal administration in humans (91-93). Although these studies indicate that the rectum and sigmoid colon are most likely to be the sites where HIV is retained, semen/viral surrogates could still reach as far as the splenic flexure (transverse colon to descending colon transition). Cell-free and cell-associated surrogates also appear to have similar distribution patterns (93). Still, a recent investigation by McElrath *et al*. (94) suggests that the rectum may be more susceptible to HIV infection than the colon due to the increased expression of the CCR5 co-receptor on rectal macrophages as compared to those in the colon. These findings were further supported by another study (95), which defined a dominant macrophage population expressing DC-SIGN in human colon and rectum. Moreover, lymphoid nodules are in higher numerical density in the rectum than elsewhere in the large intestine, thus reinforcing the higher susceptibility of the rectum to HIV transmission (96).

2.4. Rectal microbicides

2.4.1. Past and current status

The past twenty years of microbicide research have been mainly focused on the prevention of vaginal heterosexual HIV transmission. Indeed, rectal microbicide development has been in the shadow of vaginal microbicides but increasing HIV infection numbers among MSM over recent years, evidence of higher rates of transmission through the rectal route, and the noticeable interest of MSM in the concept of microbicides triggered an increasing awareness of the field (97). Of course, data obtained from vaginal microbicide studies has been of undeniable relevance and provided a solid ground for the development of rectal products. It is not surprising then that most of the products tested so far as rectal microbicides have been previously evaluated for preventing vaginal HIV transmission.

First pre-clinical studies were mainly focused in the safety assessment of non-specific microbicides developed for vaginal use, namely products containing nonoxynol-9 (N-9) (98, 99). N-9 is a non-ionic surfactant still used as an over-the-counter (OTC) vaginal spermicide and one of the first vaginal microbicide candidates. Its possesses a non-specific mechanism against HIV leading to viral disruption (100). The first rectal microbicide Phase 1 clinical trial – the HIV Network for Prevention Trials (HIVNET)-008 study – was conducted with a N-9 gel and studied not only its safety but also acceptability (101, 102). However, poor effectiveness results for this compound when tested as a vaginal microbicide, including the ability to enhance viral transmission (103), discouraged further development of N-9 products as rectal microbicides. Led by these disappointing outcomes, safety became emergent in the field and is now considered a go-no-go condition. Again, following the storyline of vaginal microbicide candidates, other surfactants (*e.g.*, C31G) (104), buffering agents (*e.g.*, BufferGel) (105) and polyanionic sulfated polymers (*e.g.*, PRO 2000, cellulose sulfate, carrageenan) (50, 106) have also been tested in pre-clinical studies as rectal microbicides but with little success and, again, suffering from poor results of these candidates in vaginal microbicide clinical trials (107-110).

From these disappointing results with early-generation unspecific candidates, the field of microbicides moved towards the use of potent ARV agents, much like those used in therapy (100). These compounds present high specificity against HIV, thus potentially being active at levels where toxicity to epithelial barriers and local microbiota are lower. The promising results of the Phase 2b CAPRISA 004 trial testing a 1% tenofovir (TFV) vaginal gel established in human proof-of-concept for microbicides (111) and boosted research in ARV drug-based microbicides, not only for vaginal but also for rectal use. Different ARV drug classes are being tested *in vitro* for their ability to protect rectal tissue explants from HIV infection, namely fusion inhibitors (*e.g.*, L´644 (112)), reverse transcriptase inhibitors (RTI; *e.g.*, TFV, UC781, dapivirine (113-115)) and protease inhibitors (*e.g.*, saquinavir (116)), either alone or in combination. Paralleling current ARV drug therapy, some microbicide combinations have been proved more potent than single drug use while allowing reducing the possibility of drug resistance, thus reinforcing the potential benefit of combination rectal microbicides (114, 117). Furthermore, various candidates have moved into non-human primate (NHP) studies and compounds such as cyanovirin-N (118), maraviroc (MVC) (119, 120), TFV (120, 121) and MIV-150 (122, 123) formulated as rectal gels were found safe and effective in preventing SIV or simian/human immunodeficiency virus (SHIV) transmission upon rectal challenge. Although humanized mice models have been less frequently used, the efficacy of rectally-applied MVC gel (124) and 1% TFV in phosphate buffer saline (PBS) (125) was also demonstrated using these models. More recently, a humanized mice model was also used to ascertain the intrarectal drug dose of TFV disoproxil fumarate (TDF) and emtricitabine (FTC) required to provide protection against HIV transmission (126).

Two Phase 1 clinical trials have been performed in order to test gel products containing either 0.1% or 0.25% UC781 (RMP-001 study) (127), or 1% TFV (RMP-02/MTN-006 study) (128), which have been previously tested as vaginal microbicides (**Table 2.2**). In the case of the UC781 gels, formulation issues dictated the premature ending of the clinical development of this compound; as for the 1% TFV gel, the RMP-02/MTN-006 study showed that the formulation previously tested for vaginal administration was not completely safe or even acceptable for rectal use, thus stressing the need for specific formulation of rectal microbicide candidates. A

subsequent Phase 1 study (MTN-007) testing a reduced glycerin version of the 1% TFV gel showed that the new product was safe and acceptable for rectal use (129).

Following the encouraging results of the MTN-007 study, a Phase 2 clinical trial (MTN-017) was conducted (130). In MTN-017, participants were enrolled to each of three regimens for eight weeks: daily reduced glycerin 1% TFV gel, reduced glycerin 1% TFV gel before and after sex, and oral TDF/FTC (Truvada®). Both rectal regimens presented similar safety profiles when compared to oral TDF/FTC. However, results for adherence and product use likelihood were favorable for the intermittent rectal gel and daily oral PrEP, while daily use of rectal gel was less acceptable.

Also, the Combination HIV Antiretroviral Rectal Microbicide (CHARM) program was set to develop and evaluate both single and combination ARV compounds as rectal specific microbicides. Two Phase 1 studies, CHARM-01 and CHARM-02, assessed the safety, acceptability, PK/pharmacodynamics (PD) correlation and distribution of the original (vaginal) 1% TFV gel, the reduced glycerin 1% TFV gel, and a rectal specific 1% TFV gel. Overall, both formulations of reduced glycerin 1% TFV gel and rectal specific 1% TFV gel were found safe and acceptable. The original 1% TFV vaginal gel was associated with minor adverse effects, higher systemic drug exposure and higher colonic distribution when compared to the other two formulations. This last phenomenon was explained by the higher osmolality of the vaginal TFV gel, which was implicated in higher fluid drawing to the colonic lumen and thus allowing the upwards gel spreading, as assessed by SPECT/CT (131, 132).

A third study, CHARM-03, assessed the safety, acceptability and PK/PD profile of both oral MVC and a rectal specific MVC gel (133). The results were not yet released but preliminary data revealed that both oral MVC and the rectal specific MVC gel were found safe and well tolerated. However, both products were ineffective in preventing viral infection in colorectal explants (134). The Project Gel study investigated rectal microbicide safety, adherence, and acceptability in young MSM from ethnic minorities (135). Firstly, participants tested a placebo gel during RAI in an acceptability and adherence trial. Next, safety of reduced glycerin 1% TFV gel and matched placebo were assessed. Once again, this study confirmed the safety and acceptability of reduced 1% TFV gel among these men.

Table 2.2. Properties of some products used in completed or ongoing rectal microbicide clinical trials.

Microbicide product	Clinical study	Excipients [(a)]	pH	Osmolality (mOsm/kg)	Refs
0.1% or 0.25% (*w/w*) UC781 gel	RMP-01	Carbomer 974P, methylcellulose, glycerin, purified water, methylparaben, propylparaben	5.2	N.A.	(127)
1% (*w/w*) TFV gel	RMP-02/MTN-006	Glycerin (20%), hydroxyethylcellulose (2.5%), methylparaben (0.18%), propylparaben (0.02%), purified water (75.23%), ethylenediaminetetraacetic acid (EDTA; 0.05%), citric acid (1%), sodium hydroxide, diluted hydrochloric acid	4.5	3111	(128)
Reduced-glycerin 1% (*w/w*) TFV gel	MTN-007	Glycerin (5%), hydroxyethylcellulose (2.75%), methylparaben (0.22%), propylparaben (0.024%), Purified water (90%), EDTA (0.05%), citric acid (1%), sodium hydroxide, diluted hydrochloric acid	4.6	836	(129)
Rectal-specific 1% (*w/w*) TFV gel	CHARM-01	Glycerin (5%), hydroxyethylcellulose (3%), methylparaben (0.22%), propylparaben (0.05%), purified water (90%), EDTA (0.05%), citric acid (1%), sodium hydroxide, diluted hydrochloric acid	7	479	(136)

(a) Concentrations are indicated in brackets if available; N.A. – not available.

More recently, a Phase 1 study, MTN-014, evaluated the drug-transfer pattern between rectal and vaginal tissues of the reduced glycerin 1% TFV gel used in the MTN-017 study upon vaginal and rectal administration, respectively, in fourteen HIV-negative women. This crossover trial was planned to assess the possibility of dual protection with a gel administered by a single route (rectal or vaginal). However, the results were disappointing and indicated that poor distribution between the two compartments occurred (137).

In addition to the previous, other studies are ongoing or planned to start in the near future. The DREAM 01, an early Phase I study, will assess the safety, acceptability, drug concentration and susceptibility of colorectal explants to HIV infection of three formulations of a TFV enema with different osmolality values. The study is planned to be concluded by the end of 2018 (138). The MTN-026 Phase I

study will evaluate the safety and acceptability of a rectal dapivirine gel (0.05%) in healthy HIV-uninfected men and women (139). In another Phase I study, MTN-033, the same dapivirine gel will be administered intrarectally using a vaginal applicator or a phallic device and the PK of dapivirine will be assessed (140). MTN-035 study will test the acceptability, tolerability and adherence of tentative placebo rectal microbicides administered to young MSM and transgender women in three different dosage forms: a rectal douche, a fast-dissolving rectal tablet and a rectal suppository (141). A Phase I clinical trial, MTN-037, will assess the safety and PK of a rectal gel that combines MIV-150 and zinc acetate in a carrageenan gel. This gel was designed to be used as a dual compartment (rectal and vaginal) gel in order to protect against HIV-1, HSV-2 and HPV (142). Finally, The Phase I clinical trial MTN-039 will evaluate the safety and PK of an insert containing elvitegravir after administration to men and women engaged in RAI (143).

2.4.2. Formulation considerations and product design

The main purpose behind the formulation of rectal microbicides is to provide adequate vehicles that can properly deliver an active pharmaceutical ingredient (API) to the site of action for a defined period of time in order to elicit protection from HIV infection. The development of rectal microbicides is supported by preformulation studies common to the development of most medicines, with the objective of characterizing APIs, namely regarding: (i) purity, (ii) physical and chemical properties (solubility, crystallinity, melting point, MW, particle size and shape, hygroscopicity and water content, acid/base ionization, polymorphism, mechanical behavior, etc.), (iii) organoleptic properties (color, odor, taste), (iv) stability in different states (dry, dispersed, dissolved) and at different pH values, (v) forced degradation studies under extreme conditions (light, heat, pH, oxidative stress), (vi) compatibility studies with excipients/materials and/or other APIs, among others (144). The previous is not an exhaustive list and may not be applicable to all compounds. At the same time, additional properties may be required depending on API. Further, the obtention of data specifically related with microbicides (*e.g.*, solubility and stability in anogenital biological fluids and tissue homogenates) might also be pursued (145).

The combination of antiviral drug candidates with different excipients should allow producing safe, effective, stable and acceptable drug products. Some formulation development algorithms have been developed to guide microbicide formulation, but are mainly concerned with vaginal application (146-148). Indeed, the development of such rectal microbicide formulations remains challenging and it is difficult to detail on the ideal product. Nevertheless, there are different aspects of formulation that appear to be critical and are detailed in the following.

2.4.2.1. Rectal lubricants and rectal drug products: lessons learned

The use of OTC lubricants during sexual intercourse is a common and frequent practice among both men and women, particularly in populations engaging in URAI (149, 150). In some cases, namely in regions where access to these products is limited, other fluids (*e.g.*, saliva, egg white, yoghurt, soft paraffin) have also been used as surrogates (151). These practices are easily understandable as the rectum does not self-lubricate before or during intercourse, contrasting with the vagina. Hence, lubricants allow for more comfortable and non-traumatic anal sex. The concomitant use of lubricants and male condoms may also be beneficial since decreased breakage rates (around 7-fold less than for no lubricant) have been reported (152).

The wide use and potentially protective effects of these products (*i.e.*, due to decreased shear during intercourse) provide an important rationale for the development of rectal microbicides and may even facilitate their future acceptance if formulated as lubricant-like products. Indeed, commercially available vaginal lubricants have been considered in the past as favorable vehicles for the incorporation of microbicide drugs. The Food and Drug Administration (FDA) does not regulate lubricants as medicines but as medical devices, thus excusing most of the extensive pre-clinical and clinical testing required for drug products (153), similarly to the European Medicine Agency (EMA). Lack of strict regulation is also noticeable in other regions of the world and, thus, performance of lubricants, particularly their safety, is not fully known (154). Even so, data deriving from recent *in vitro* and *in vivo* studies of lubricants provided important hints on the desirable

attributes of rectal products and, more important, perils related with the influence of formulation issues on the enhancement of HIV transmission and that should be considered when developing microbicide products (155, 156). Several of these aspects are addressed further in this manuscript. Of particular importance is safety namely due to the potential long-term use of a rectal microbicide product. Products containing N-9 were frequently used by MSM for their lubricant ability, being inclusively considered early on as potential rectal microbicide candidates (157). However, studies conducted with N-9 OTC products demonstrated the transient but potential hazardous effects of this detergent, namely rectal epithelial damaging (**Figure 2.5**) and consequential enhancement of HIV transmission (158), much in line with what had been previously demonstrated for its vaginal use (103, 159).

Alongside N-9, other ingredients frequently present in lubricant products (*e.g.*, polyquaternarium-15, clorohexidine) may have the potential to increase HIV replication as demonstrated *in vitro* (160, 161). Some lubricants may also deplete native microbiota, thus compromising the integrity of this potentially helpful barrier against pathogens. Further, differences in the colorectal and female genital safety profiles of some lubricants have been shown *in vitro* (161). These observations reinforce that specific rectal microbicide products are required and translation from vaginal data is not advisable.

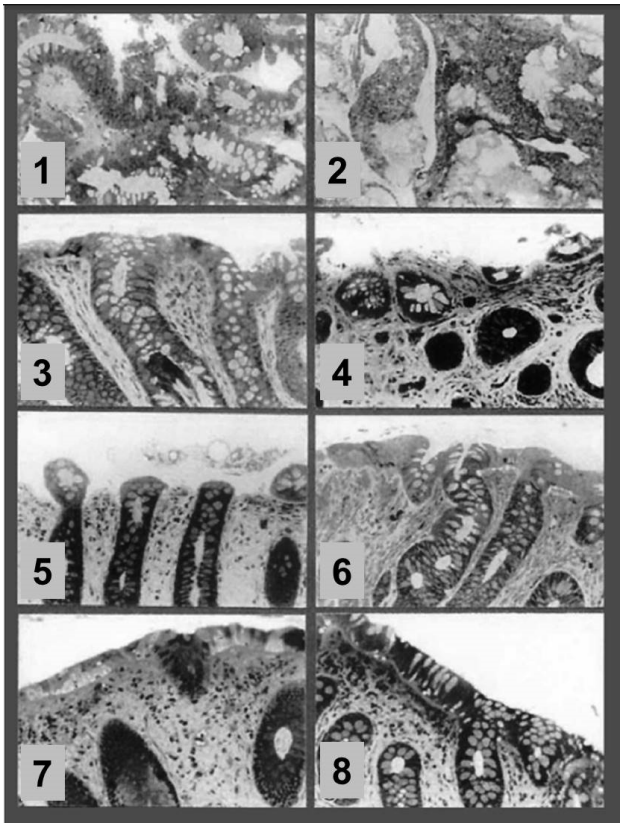

Figure 2.5. Representative photomicrographs of rectal lavages and biopsy specimens of 18 participants at different time points (15 min, 2 h and 8 h) following rectal application of a 2% N-9 gel (K-Y® Plus, Ortho-McNeil-Janssen Pharmaceutical). All samples were stained with toluidine blue and images taken at ×96 magnification. (1) Rectal epithelium in lavage specimen collected 15 min following insertion of the N-9 gel, revealing sheets of gut epithelium composed of columnar and goblet cells. (2) Degraded cells in lavage specimen taken 2 h post N-9 gel insertion. (3) Baseline biopsy of rectal epithelium showing the typical simple columnar epithelium forming crypts of Lieberkühn and presenting goblet cells. (4 and 5) Biopsies collected 15 min post-insertion of N-9 gel and evidencing some areas where the epithelium was separated, or partially separated, from the underlying submucosa (4); in some biopsies the epithelium directly opposed to the rectal lumen was not present (5). (6 and 7) Biopsies collected 2 h post N-9 treatment and showing intact epithelium. (8) Biopsy taken 8 h post N-9 treatment evidencing indistinguishable tissue architecture from that of baseline. Reprinted with permission from reference (158), Copyright (2004), with permission from Elsevier.

2.4.2.2. Dosage forms

The rectal administration of pharmacological agents can be achieved by using several dosage forms as noticed above. However, only a few have been explored for developing rectal specific microbicides, namely suppositories, gels and enemas. Hydrophilic gels seem to be preferred by formulation developers as these are easily manufactured and affordable, despite potentially having stability problems and limited incorporation ability of hydrophobic compounds (162). Enemas share basic characteristics with gels, being consistency their most striking difference. The choice of this liquid dosage form is intimately related with the popularity of rectal douching among MSM engaging on RAI prior to or following sexual intercourse (163-165). Suppositories have a long history of rectal usage for drug delivery but acceptability problems related with cultural issues and misconceptions may undermine their widespread use (166). Despite being solid dosage forms with low water content, which may abbreviate drug stability issues, they are often designed to melt at body temperature by mixing excipients with different melting temperatures. However, their storage may be troublesome in tropical or otherwise hot regions of the globe where microbicides are most needed.

Gels, in particular, enjoy good acceptance among users despite disadvantages such as product leakage and messiness upon administration, requiring an applicator for delivery, and coital dependence (167). For instances, Carballo-Diéguez *et al*. (168) compared the acceptability of a lubricant gel inserted using an enema bottle (35 mL per administration) *versus* a 8 g non-medicated suppository (**Figure 2.6**), used in up to three RAI occasions by 77 MSM. Overall, preference ratings for the gel were far superior to those for the suppository as perceived by both the participants and their partners. However, the large size of the studied suppository may have influenced acceptability, suggesting that smaller formulations, closer to those commonly used in therapy (2-3 g), may be preferable. Previous lubricant use patterns were not assessed, which could have introduced some bias in the overall appraisal of obtained results. In a more recent study enrolling males and females engaged in URAI, Pines *et al*. (169) assessed the acceptability of three OTC rectal products formulated as distinct dosage forms: (i) a solution filled in an enema bottle (Normosol®-R, Hospira, Inc., 125

mL/administration), (ii) a lubricant gel (Pre-Seed®, Lil' Drug Store Products, Inc., 4 mL/administration) delivered using a pre-filled vaginal applicator, and (iii) a suppository (Tucks™, McNEIL-PPC, Inc., 1.4 g/administration) commonly used in hemorrhoids relief. The lubricant gel scored higher overall acceptability rates as compared to the other two dosage forms, namely the small size suppository. Previous experience with lubricant gels (over 85% of participants), however, may also help explaining obtained results.

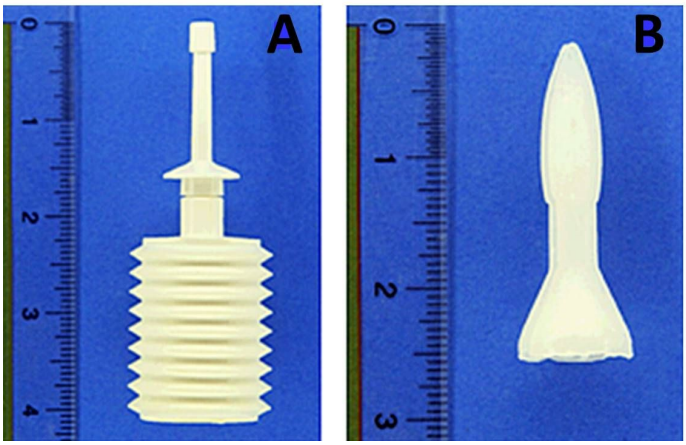

Figure 2.6. Placebo products used in the acceptability study by Carballo-Diéguez et al. (168). (**A**) Lubricant gel in an enema bottle and (**B**) suppository ("rectal rocket"). Scale bars in images are in inches (1 inch=2.54 cm). Reprinted with permission from reference (168), Copyright (2008), with permission from British Medical Journal Publishing Group.

2.4.2.3. Choice of excipients

Besides functional properties related with specific dosage forms, excipients used in the formulation of microbicides need to ensure maximum activity and stability of APIs, while presenting no significant toxicity at used amounts/concentrations. Most of the products tested in clinical trials as rectal microbicides, and therefore their excipients, have been directly transposed from those used for vaginal administration (**Table 2.2**) (127-129, 136). Also, the choice of excipients for microbicide formulation has been traditionally based on functionality rather than safety, and mostly based on

past experience of their use in rectal drug products. Many of these excipients are included in the FDA *"Inactive Ingredient Database"*, as well as used concentrations according to the route of administration and/or dosage form. However, the relevance of these data is limited since it is based on products used with therapeutic purposes and usually for short-term usage, contrasting with the preventive aim and potential long-term usage of rectal microbicides. Also, the inclusion of an excipient in this list does not immediately rules out toxicity issues. The attention of regulatory agencies is indeed focused on the safety of products and excipients used in therapeutic products (170). Further, FDA guidelines for the safety evaluation of excipients seem to be very broad in order to adequately attest the suitability of an excipient for rectal microbicide formulation (171).

Most preclinical safety/toxicity studies have focused mainly on APIs or the overall product formulation, while the evaluation of the toxicity of single excipients for rectal microbicides has been mostly neglected. For example, carbomers are popular and quite useful hydrophilic gelling agents in the formulation of rectal products (172). Despite being considered non-toxic and non-irritant excipients, with a long-term record of safety (173), Abner *et al*. (50) showed that a placebo carbomer-based gel caused a reduction of roughly 43% on the viability of human colorectal explants, contrasting with the low toxicity of hydroxyethylcellulose (HEC) and methylcellulose placebo gels. Even if relevant, the previous results may have been influenced by the presence of other excipients, in various degrees, thus limiting the usefulness of obtained data.

Ethylenediaminetetraacetic acid (EDTA) is often used in rectal microbicide formulation (**Table 2.2**). Despite that, this chelating agent has been previously shown associated with enhanced HIV-1 replication in ectocervical explants (161) and HSV-2 vaginal infection in mice in a dose dependent manner (174). In the case of rectal use, it is known that EDTA allows increasing drug permeability (175), presumably due to calcium chelation and impairment of intercellular tight junctions as observed *in vitro* (176). Although the effects of such events on HIV transmission cannot be directly inferred, such potential epithelial barrier disruption may be deleterious. Further, EDTA showed to be toxic to the colorectal Caco-2 cells at concentrations of 20 nM and 50 nM, with a decrease of 76% and 52%, respectively, on cell viability after 6 hours of exposure to this absorption enhancer (177). Nevertheless, a recovery

back to 93% and 82% on cell viability, respectively, was observed 24 hours after replacement of EDTA solution by fresh culture medium.

Suppository bases are generally regarded as safe, including for pediatric use (178), but the specificities of rectal microbicides may limit their use in the formulation of safe preventive products. Again, very few data are available and extensive work is required. For instances, different commercially available mixtures of glycerides (Witepsol® H15, H12 or H19, and Suppocire® AP), and PEG have been shown to affect mucosal integrity upon rectal delivery to rats (179, 180). Moreover, while the effect of some bases was reversible after 24h (*e.g.*, Witepsol® H15), mucosal damaging was further exacerbated by others after this time period (*e.g.*, PEG 1540/6000). In another study using a rabbit model, researchers also showed that PEG- and triglyceride-based materials caused irritation of the rectal mucosa after subchronical exposure, whereas monoglyceride- or fatty acid-based materials were less irritating (181).

While the impact of excipient toxicity on HIV transmission or other pathogens infection may not be easily inferable, it seems reasonable that this matter should be considered and preliminarily tested as some compounds have been demonstrated to enhance rectal penetration or absorption of viral particles. For example, PEG monohexadecyl (or cetomacrogol 1000), a non-ionic surfactant used in suppository formulations, was shown to increase the absorption of Felix virus from the rectum of healthy rabbits (182). Overall, the above presented examples emphasize the necessity of toxicological evaluation of excipients intended for microbicide use and, ideally, the establishment of a specific database of safe (and hazardous) ingredients.

2.4.2.4. Volume to be administered

There is no standard for the amount of a liquid or semisolid microbicide product that should be administered rectally. The same applies to dosage forms that liquefy upon delivery (*e.g.*, fatty base suppositories). The volume should be enough to protect the entire area that may potentially be used for HIV transmission, while abbreviating safety issues and being acceptable by users. Values around 3.5-4.0 mL arising from clinical practice (where only lower rectum action or absorption is usually

desired) and vaginal microbicide trials have been frequently used in the clinical testing of rectal microbicide gels (127-129). However, recent studies suggest that a rectal microbicide should cover the entire rectosigmoid and descending colon, ideally up to the splenic flexure, in order to provide complete protection against HIV (91-93, 132). Achieving these distances will definitely require for higher volumes of a gel or a liquid, eventually in the order of those administer as enemas for IBD treatment (40-100 mL) (33). However, two studies conducted by Hendrix and colleagues (91, 92) evidenced that volumes as low as 10 mL of a lubricant gel (K-Y® Jelly, Johnson & Johnson) were able to successfully reach the sigmoid and the descending colon in humans (**Figure 2.7**). The administration of the gel was followed by simulated anal intercourse with an artificial phallus, which may have also contributed to the observed distribution of the gel. Nevertheless, and since rheological properties of different products (including their dilutions with rectal fluids) may influence spreading and distribution patterns, as previously described for vaginal microbicides (183), these volumes should be seen as merely indicative. More recently, the same group showed that 3.5 mL of radiolabeled (1 mCi ^{99m}Tc-DTPA) universal placebo hydroxyethyl cellulose gel was able to provide similar surface coverage as a higher volume (10 mL). Both volumes were well tolerated and high acceptability levels were reported (184).

Another human trial showed that 125 mL of a isoosmolal enema (Normosol®-R; 295 mOsm/kg) had a greater distribution and tissue retention pattern when compared to hyperosmolal (Fleet® Phosphate, Fleet Laboratories; 2,100 mOsm/kg) or hyposmolal (distilled water; 0 mOsm/kg) enemas as evaluated in the same study. The isoosmolal enema distributed through the distal colon and in some cases reached the splenic flexure, whereas the hyperosmolal was confined to the rectosigmoid (185). Water uptake or exudation by the mucosa as determined by electrolyte concentration may be implied in these findings.

As acceptability is concerned, a single study conducted by Carballo-Diéguez *et al*. (186) assessed the influence of escalating volumes (5-50 mL) on the willingness of twenty men engaged in URAI to use a lubricant originally intended for vaginal administration (FemGlide®, Coopersurgical, Inc). These researchers found that up to 35 mL of gel were acceptable to the majority of participants, although smaller volumes were preferred (only 5 mL were completely acceptable). Increased

leakage and messiness were reported beyond 35 mL. Further, perceived comfort was variable when the gel was used with or without sexual intercourse. In the case of enemas, the volume of 125 mL of the isoosmolal enema aforementioned was overall accepted by the subjects included in this group (n=9). Therefore, an ideal rectal microbicide product should allow being administered in a relatively wide range of volumes while maintaining safety and efficacy.

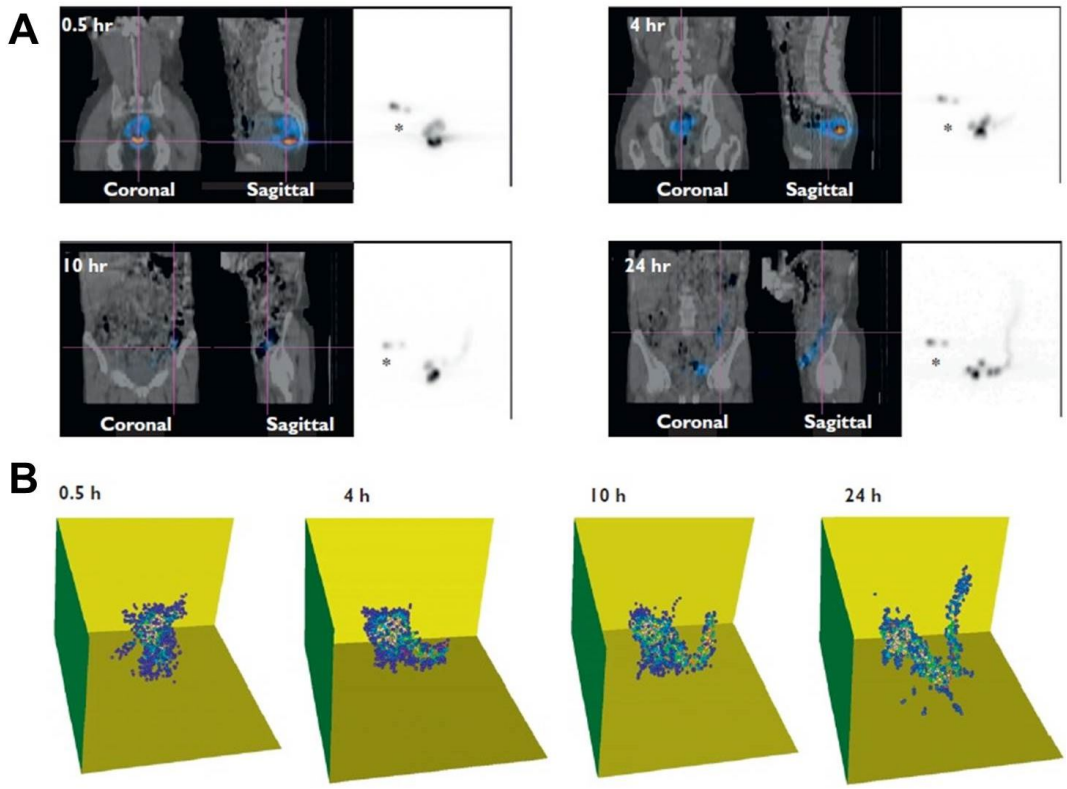

Figure 2.7. Representative spatial distribution of a microbicide surrogate in the lower GIT upon rectal administration (92). The participant received 10 mL of the commercially sexual lubricant K-Y® Jelly labeled with 100 nm 99mTc particles, followed by simulated intercourse with an artificial phallus. Single photon emission computed tomography (SPECT) and computed tomography (CT) imaging was performed at different time points after administration. The microbicide surrogate migrated in a retrograde fashion into the rectosigmoid colon. At 4 h post-administration, imaging signal was evident at the distal descending colon, reaching the splenic flexure 24 hours later. This distribution pattern was observed for two other subjects using magnetic resonance imaging (MRI). (**A**) SPECT/CT fusion imaging. The microbicide surrogate is shown in blue. The co-registered CT image is in grayscale. In each panel, the coronal section is on the left, the sagittal section in the middle and maximum intensity projection (SPECT only) in the coronal plane on the right. (*) indicates radiotracer on body surface used for anatomical reference. (**B**) Random samples (1,000) taken from each SPECT image and plotted in three dimensions. The blue-green-orange color shows the signal intensity in increasing order. The red curve shows the center-line found through 3-D curve fitting [green = left (sagittal); yellow = back (coronal); dark yellow = bottom (transverse)]. Scale changes with each scan to maximize detail. Adapted from (92), Copyright (2012), with permission from the British Pharmacological Society.

2.4.2.5. pH and buffer capacity

Fluids present in the rectum of healthy individuals are known to possess pH close to neutrality and low buffer capacity (16) and, hence, administration of a product may significantly impact final proton activity. Variations in pH can impact drug absorption and, most importantly, extreme values may potentially lead to irritation, discomfort or even pain (187). The effects of changes to the normal rectal fluid pH on the epithelial integrity, colorectal microbiota and HIV transmission are not well understood. Thus, it seems advisable that microbicide products maintain the pH at values around 7-8 since extreme values may become problematic, particularly with long-term use (188, 189). Some data, however, suggest that a wider pH range may still be safe. For instances, Good™ Clean Love (Good Clean Love, Inc.), an aqueous lubricant with pH around 4.8, was shown to maintain epithelial viability of Caco-2 cells and the integrity of colorectal explants (161). Also, to date, most gels used in rectal clinical trials (**Table 2.2**) have been formulated for vaginal administration, with pH values around those normal for the vagina (around 4-5). In the cases of RMP-01 and MTN-007 trials, no adverse effects or changes in epithelial integrity were reported up to a maximum of seven days of product use (127, 129). However, no systematic *in vitro* or *in vivo* testing of the safety for extended use of low pH products, namely on the distal GIT, has been performed so far and further work is required. Finally, a recent draft position paper from the World Health Organization (WHO) recommends that the pH for lubricants used concomitantly with condoms during rectal intercourse should be in the range of 5.5 to 7, with higher pH values being discouraged due to potential poor product preservation (190).

2.4.2.6. Osmolality

There is a growing body of evidence that osmolality can impact significantly the safety of rectally applied products. For instances, the adverse effects observed during the RMP-02/MTN-006 clinical study (7-day rectal use of 1% TFV vaginal gel) were associated with the hyperosmolality (3,111 mOsm/Kg) of tested formulation (128). These observations add up to the existing *in vitro* and *in vivo* studies using

commercially available lubricants, showing that hyperosmolal gels can indeed cause rectal epithelial damage (160, 191, 192) and potentially enhance HIV infection risk (193). The same behavior was observed for an hyperosmolal enema rectally administered (185). In particular, the high content in glycerin, a common excipient in rectal products, is usually responsible for these high values of osmolality. The WHO advises that the osmolality of lubricants (either vaginal or rectal) to be used concomitantly with condoms should be preferably equal or less than 1,200 mOsm/kg (190). Levels of glycerin, propylene glycol, or a mixture of glycols are recommended to be maintained at or below 9.9% (*w/w*), 8.3% (*w/w*), or around 8.3% (*w/w*), respectively, in order to achieve the previous. Results from an *in vitro* study showed that 1% TFV gels containing either 5% or 20% glycerin (836 and 3,111 mOsm/kg, respectively) presented different toxicity profiles (**Figure 2.8**) (194). Epithelial fracture and sloughing in polarized colorectal explants was only observed for the 20% glycerin formulation, while other characteristics of the vaginal formulation, namely rheological profile, drug release profile, tissue permeability and *in vitro* activity, were maintained (194). The 5% glycerin gel was further tested in the MTN-007 Phase 1 clinical trial, showing to be generally tolerable and safe (129). Even so, some adverse events were still observed but with lower incidence and severity than those observed in the RMP-02/MTN-006 study (128). A recent Phase 1 study, CHARM-01, evaluated the impact on mucosal safety of three different formulations of 1% TFV gel presenting different osmolality values: the 20% glycerin original vaginal gel, and two 5% glycerin formulations, namely the reduced glycerin gel and the rectal specific gel (**Table 2.2**) (195). All the three formulations were found safe. However, these results should be interpreted carefully since the safety profile of the original vaginal gel was ascertained based on one single exposure, while the reduced glycerin gel and rectal specific gel were administered for seven consecutive days. Curiously, and despite the osmolality of the reduced glycerin gel being half of the rectal specific gel, no differences were found regarding their safety profiles (131).

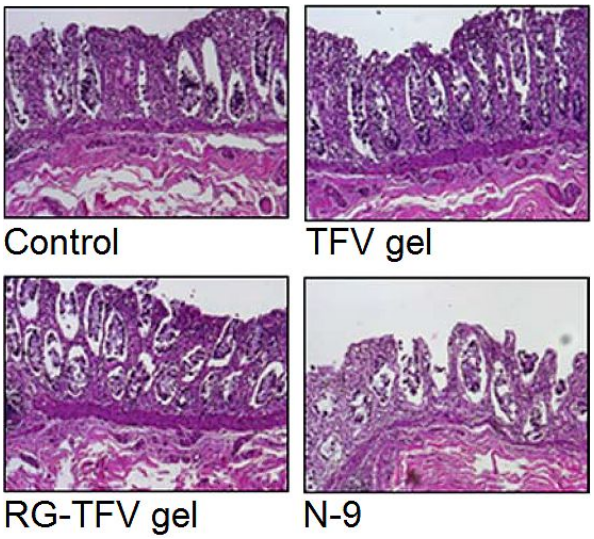

Figure 2.8. Representative tissue histology after exposure of polarized colorectal explants to a 20% glycerin TFV gel and a reduced glycerin (5%) tenofovir gel (RG-TFV). Colorectal explants were exposed overnight to 1:5 dilutions of either 5% or 20% glycerin gels, or a 2% N-9 gel (Gynol II, Ortho-McNeil-Janssen Pharmaceutical). Untreated explants were used as control. Colorectal epithelium was intact after exposure to the 5% glycerin TFV gel while the epithelium was fractured or sloughed off after exposure to the 20% glycerin TFV gel. Adapted from (194), Copyright (2012) with permission from the Oxford University Press.

Even if the 1,200 mOsm/kg upper limit has been defined as acceptable, it still seems quite high and the WHO recognizes that osmolality values above 380 mOsm/kg already represent a potential hazard, being near-isoosmolal products preferential (190). *In vitro* and animal *in vivo* data obtained by Dezzutti *et al*. (161) and Wang *et al*. (188) seem to provide evidence for this position. Another question relates with hypoosmolal products. Even if there is a lack of consistent data supporting either safety or not, the former study showed that one lubricant with 13 mOsm/ kg (Slippery Stuff®, Wallace O'Farrell, Inc.) led to epithelial loss in colorectal explants, although this might have been induced by other factors (161). Thus, caution is advised in the case of hypoosmolal formulations.

2.4.2.7. Stability and shelf-life

Like for other pharmaceutical products, stability testing and assignment of shelf-life are essential in order to assure efficacy and safety of a microbicide, and comply with regulatory requirements leading to marketing approval. Microbicides will probably require worldwide distribution, storing and use, namely in tropical settings where extreme temperatures and relative humidity are observed. Also, low and middle income regions may not have the necessary infrastructures to allow cold-chain distribution. In these cases, solid dosage forms such as tablets offer the potential for improved stability as compared to liquids, gels and suppositories (196). There are no current specific orientations on stability testing for rectal microbicides, but a FDA draft guidance for vaginal microbicide development recommends that the stability profile for a product should be performed under long-term and accelerated storage conditions (197), as described by the same agency guidelines regarding stability testing (198). The scientific literature on microbicide stability studies has been scarce and mostly related with the development of vaginal microbicides. Still, studies should not only be restricted to chemical stability but also expanded to properties that are known to influence product performance such as viscosity, mechanical properties or pH, to name a few (199, 200). A shelf-life of years for microbicides is suggested although shorter timeframes may also be considered.

2.4.2.8. Rectal applicators

With the exception of suppositories, other rectal dosage forms require the use of an applicator. Such devices should be specifically designed for rectal use and assure comfortable administration of a suitable dose, while possibly allowing the reduction of leakage and adequate product distribution inside the rectum. Rectal microbicide clinical studies conducted so far relied on single dose, prefilled applicators originally intended for vaginal administration. However, acceptability has been suboptimal, with complaints about the applicator (often perceived as too large, poor appearance), unpleasant effects attributed to its use, difficulties in handling and inadequacy for casual transportation (102, 127, 201). Carballo-Diéguez *et al*. (202)

approached the problem and set out to develop a specific rectal microbicide delivery device, comparing its acceptability with that of a vaginal applicator among MSM (**Figure 2.9**). Surprisingly, the rectal-specific applicator was not superior to the vaginal applicator thus highlights that more research is required in order to fully understand what users want from an applicator. The results from the previous study also indicated that portability, discretion, aesthetical appeal and easy-to-open packaging are desirable features of a rectal applicator (202). More recently, the same applicators were tested in the Project Gel study and the results were similar the ones reported for the first study (134).

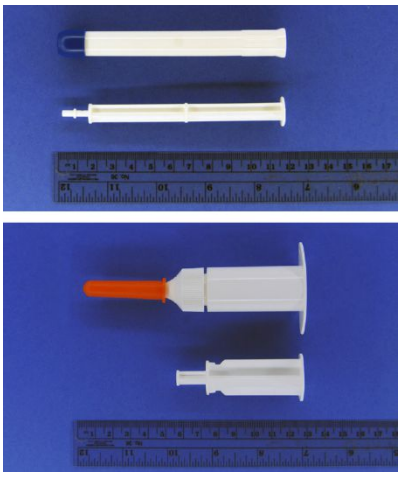

Figure 2.9. Vaginal (top image) and rectal specific (bottom image) applicators compared in a acceptability study among MSM (202). Scale bars in both images are in centimeters (top scale in ruler) and inches (bottom scale in ruler). Adapted with permission from reference (202), Copyright (2014), with permission from Springer Science+Business Media.

One relevant issue regarding applicators is the possibility to re-use them. Even if disposable prefilled applicators seem to be preferred by users, allowing the delivery of an accurate dose with faster and minimal product handling, multiple-use devices allow for price reduction (a major issue in resource-poor settings) and abbreviate ecological problems related with plastic disposal (203). Major setbacks of multiple-dose applicators are portability, contamination, in-use stability and adequate cleaning

after usage. Further, alternative materials for both single- and multiple-use applicators are also desirable, particularly to allow cost reduction. For example, cheaper paper applicators being developed for the delivery of vaginal gels (204) may also be transposable to the rectal microbicide field. Moreover, the use of applicators may not be impulsive and immediate, and interfere with the spontaneity of a casual sexual encounter, which can potentially lead to poor usage. Thus, novel or inventive products that do not require an applicator must be explored.

2.4.3. Characterization of rectal microbicide products

Alongside the preliminary assessment of API(s) physicochemical and biological properties, rectal microbicide products should complete an extensive set of studies in order to characterize the above mentioned and other critical features (188). Different algorithms, recommendations and systematic approaches have been proposed in order to guide pre-clinical evaluation of anti-HIV microbicide products but most of these only address briefly microbicides for rectal use (146-148, 205-207). This manuscript does not aim to provide a detailed description of the vast collection of possible testing procedures applicable to rectal microbicides. Still, a schematic overview of relevant pre-clinical assays is presented in **Figure 2.10**.

Technological/physicochemical characterization techniques of a microbicide product are highly dependent on the dosage form used to incorporate the API(s). In any case, products should be characterized for the above discussed properties and exceptions require justification. The importance of technological/physicochemical parameters is unquestionable and their use in the establishment of different models may be useful in predicting the performance of microbicide products in the rectum. For example, mathematical modeling may be advantageous in studying product spreading and retention in vaginal and rectal compartments, particularly at development and optimization stages (208).

Further, microbicide products are required to be compatible with condoms. Water-, glycol- and silicone-based products are usually acceptable for use with condoms, contrasting with oil-based formulations, although specific testing is required. Additional information on the subject and lubricant-condom compatibility

testing guidance has been published in 2010 and revised in 2013 by the WHO and can be readily used for microbicide development (209). Multiple testing standards and protocols are also available, namely from the International Organization for Standardization (www.iso.org) and ASTM International (www.astm.org).

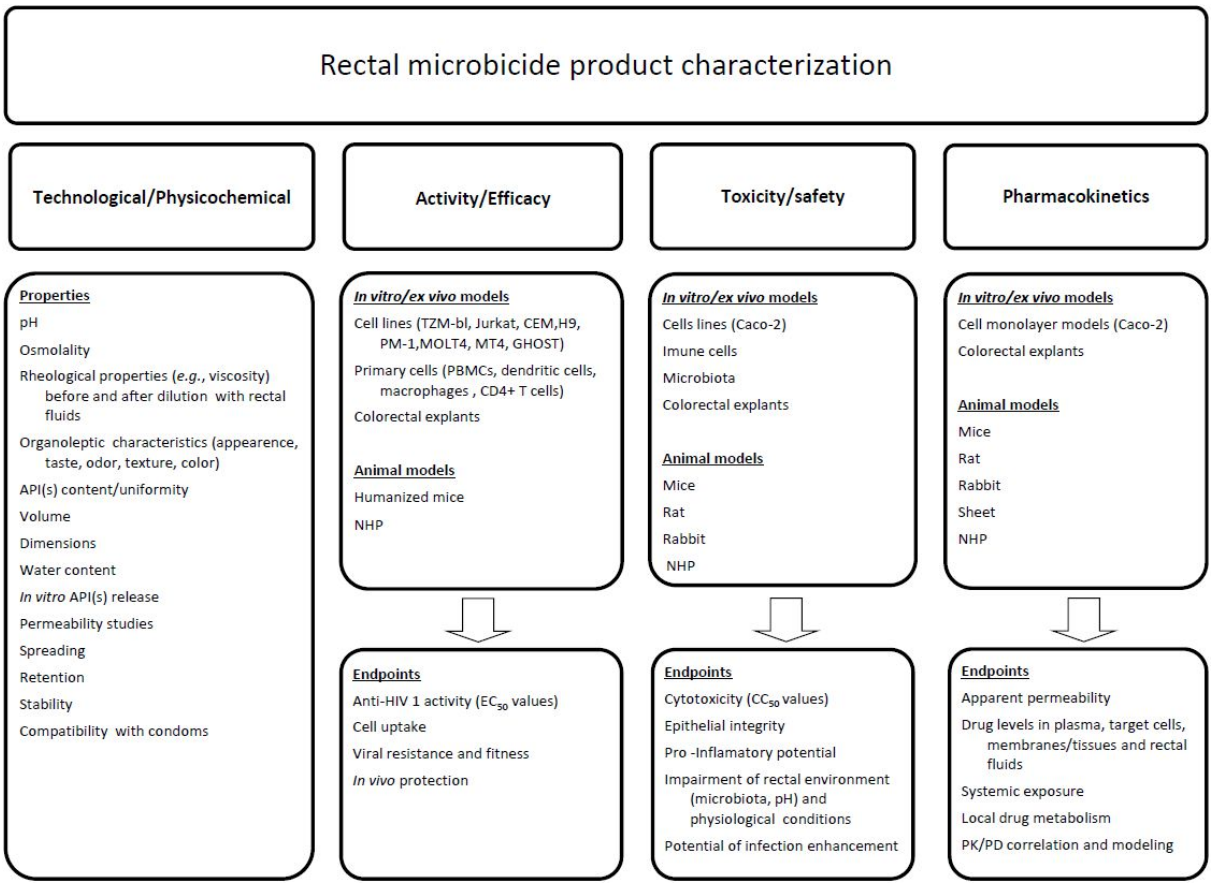

Figure 2.10. Pre-clinical testing of rectal microbicide products: technological/physicochemical, activity/efficacy, toxicity/safety and PK assessment. API, active pharmaceutical ingredient; CC50, half-maximal cytotoxicity concentration; EC50, half-maximal effective concentration; NHP, non-human primates; PBMCs, peripheral blood mononuclear cells.

Even if API(s) have been shown effective in inhibiting the virus, formulation into a microbicide product may impact activity (210, 211). Products should be sequentially evaluated in cell, tissue and animal models for activity/efficacy and toxicity/safety. The presence of different ARV compounds may further lead to synergistic or additive effects. *In vitro* activity should be evaluated against both CCR5- and CXCR4-tropic HIV-1 (and ideally against HIV-2), cell-free and cell-associated virus, and different lab strains and clinical isolates. Several primary cells or established cell lines expressing CD4 and one or both co-receptors can be used, although peripheral blood mononuclear cells (PBMCs) and TZM-bl cells are the most commonly used in initial screening. The use of CXCR4 expressing cells is usually limited to the compounds previously found to be active against CCR5-tropic viruses. The maintenance of the antiviral activity should be accessed also in the presence of natural and/or simulated seminal and rectal fluids and under rectal environment conditions. Also, colorectal explants abbreviate lack of tissue organization of most cell-based assays and allow better mimicking natural infection. Animal models susceptible to HIV (or SHIV) are reserved for late stage efficacy testing, mainly due to cost, handling and ethical restrictions, particularly relevant in the case of NHP (212). Interspecies differences at the anatomical and physiological levels require to be considered when formulating a product, namely when miniaturization or pH changes are required. In fact, these differences may impair the true value of animal efficacy studies as lack of translation of data obtained from animal models to humans has been reported, namely for humanized mice (213).

Paralleling with antiviral activity/inhibition of transmission testing, the toxicity of microbicide products to cells, tissues and microbiota, as well as their ability to modulate pro-inflammatory responses, needs to be accessed. The combination of antiviral activity and cytotoxicity data allows defining the selectivity index (SI) of an API or product, which will be invaluable in guiding through the selection of an appropriate dose (or dose range) for early clinical trials. The interactions between the microbicide product and the rectal environment (pH, microbiota, rectal ampoule contents, mucus and semen) should be clearly understood in a way that assures the activity and safety of microbicide compounds. In the case of animal safety/toxicity testing, different species have been used. Although HIV-susceptible models do not

seem essential, their inclusion may be beneficial in terms of identifying events that lead to enhanced viral transmission.

More recently, PK studies have been introduced into the field of microbicides since there is growing clinical evidence of a direct correlation between cell/tissue drug levels and protection (214). *In vitro* models, namely Caco-2 cell monolayers, provide a useful screening method to assess permeability and cell/tissue accumulation (215). However, pre-clinical rectal PK studies have been mostly performed in NHP (121, 122, 216, 217). Rectal tissues and rectal fluids (collected as lavages or by means of sponges/swabs) are used for analyzing the absorption and local distribution pattern of the microbicide compounds, while blood samples are useful in determining the extent of systemic exposure. These data and their correlation may be useful in the preliminary design of clinical trials, including statistical modeling (218). Another interesting feature in some of these PK studies is the evidence of a transference pattern of the drug between rectal and vaginal tissues following either type of administration (216, 219). Although not completely explored, the local metabolism of ARV drugs in colorectal tissues when administered topically is another issue that needs to be considered since multiple enzymes of cytochrome P450 and UDP-glucoronosyltransferase families are actively expressed in different parts of the human colon and are able to metabolize ARV drugs such as dapivirine and MVC (220, 221). Finally, one particularly neglected issue has been the effect of microbicides in insertive partners, mainly because relatively brief penile exposure is expected. However, clinical evidence indicates that there is the potential for significant local and systemic drug exposure once a microbicide gel is applied to the penis, which could lead, for example, to toxic effects (222). Thus, it seems advisable that this concern should also be considered early on pre-clinical studies.

2.4.4. User acceptability issues

Product acceptability is expected to be critical to the overall adherence and, ultimately, the effectiveness of rectal microbicides. The problem is simple: if not used consistently, microbicides cannot protect adequately from viral transmission. However, the concept of acceptability is not easy to study because it is strongly

influenced by multiple factors, including cultural and social, and depends not only on users but also on their partners' preferences. Acceptability product strongly correlates with PK and safety, which combined may influence the overall efficacy of a microbicide product (223). Early opinion in the field was not clear as to the relevance of acceptability studies prior to the demonstration of clinical efficacy or the usefulness of such findings to early pre-clinical microbicide development (224).

However, it is currently recognized that acceptability is of paramount importance in assuring protection, being intimately linked to readily perceived and subjective characteristics of the microbicide product. Features such as consistency, texture, organoleptic properties (taste, odor and color), format, size/volume to be administered, easiness to administer, package/applicator design are determinant for product acceptability. The final characteristics of a rectal microbicide can influence performance namely various in-use effects (before, during and after intercourse), frequency and timing of product use, duration of protection, lubrication, use with/without condom, changes in hygiene practices, and side effects (169, 225). Other factors, including concomitant anal and vaginal sex in the same encounter, and preferences and attitudes of partners relating to the product are important variables that need to be considered. Age, gender, economical status (intimately related to microbicide cost) are additional aspects that significantly impact patterns of product acceptability or the strength of product preferences (169). These issues impair achieving a "one size fits all" kind of product and multiple equivalent options should be considered during microbicide development. **Table 2.3** summarizes the findings of different acceptability studies regarding the rectal use of microbicide or placebo/lubricant products. In general, gels seem preferred over other dosage forms, mainly due to their acceptable organoleptic features. As discussed, volume is an important issue regarding performance but, contrasting with vaginal use, rectal administration of relatively high amounts of gels seems to be possible without affecting acceptability significantly. Moreover, safety, in-use feel and comfort are significant issues towards good acceptability. Unfortunately, it appears that a universal ideal product cannot be established from the acceptability point of view and further work in the field is required.

Table 2.3. Acceptability outcomes of the clinical evaluation of different rectal microbicide or surrogate products.

Product	Population	Acceptability main findings	Refs
N-9 gel [(a)]	MSM (n=70)	Less than half of the participants reported intention to use if approved. Some problems with gel application reported due to (vaginal) applicator design. Considered too sticky and drying out too quickly. Unappealing taste, smell, and color.	(102)
FemGlide® gel [(a)]	MSM (n=20)	Up to 35 mL of gel administration was generally acceptable. Acceptable volume was dependent on sexual activity/rest. Transparent and odorless features were acceptable. Likelihood of future use was high.	(186)
FemGlide® gel [(a)] **or PEG-based suppositories**	MSM (n=77)	Gel was overall preferred over suppository, namely in color, smell, consistency, and feeling in rectum immediately after insertion and/or 30 minutes after insertion.	(168)
0.1% or 0.25% UC781 gel [(a)]	MSM/women (n=36)	Overall well acceptable. Participants reported high intention to use if approved.	(127, 201)
TFV 1% gel [(a)]	MSM/women (n=18)	One quarter of participants liked the gel (likelihood of future use around 75%). Adverse effects reported (probably related with hyperosmolality).	(128)
Reduced-glycerin TFV 1% gel	MSM/women (n=65)	Likelihood of future use was 87% *vs.* 93% for a HEC gel.	(129)
Enema [(b)]**, lubricant gel** [(c)]**, or suppository** [(d)]	MSM/women (n=117)	Gel received highest overall acceptability score. Younger males preferred gel but older ones did not show preference among tested products.	(169)

[(a)] Originally intended for vaginal administration; [(b)] 125 mL Normosol®-R filled in an enema bottle; [(c)] 4 mL Pre-Seed® in pre-filled vaginal applicator; [(d)] Tucks™ (1.4 g).

2.4.5. Manufacturing, distribution and affordability

Rectal microbicides should be reasonably priced (or even ideally free), particularly in resource-poor countries where most of the individuals at high-risk of infection live. The manufacturing process should comply with current good manufacturing practice (cGMP) and allow obtaining quality products at minimal prices. Formulation strategies, in particular, play a decisive role in the selection of suitable excipients at reduced costs and manufacturing techniques that should ideally be widely available, readily transposable among different sites or different scales,

and require inexpensive equipments. Dosage forms so far being developed as rectal microbicides, namely gels, are quite affordable and easily produced in large-scale batches. For example, the 1% TFV vaginal gel produced in the USA and used in the RMP-02/MTN-006 clinical trial (128) may potentially be commercially available at an estimated cost of US $0.13 per dose (considering a multi-use tube with a reusable applicator), with prices rising to near US $0.20/dose or US $0.66 when considering the use of paper applicators or pre-filled single-use plastic applicators, respectively (226). In particular, prices for the 1% TFV gel as delivered by reusable applicators or even single-use paper applicators may be considered reasonable in order to assure cost-effectiveness at resource-poor countries, depending on the degree of protection provided, the available support from governmental and non-governmental agencies involved in the fight against HIV/AIDS, and partnership with the pharmaceutical industry (227). Assessing users' willingness to pay for microbicides is also important. For example, a recent acceptability study using conjoint analysis, *i.e.*, considering different hypothetical product characteristics and measuring their value to users, found that prices of around US $0.30 per dose were regarded as affordable by South American MSM willing to use rectal microbicides (228). Moreover, further strategies to reduce costs are required and include, among others, the transfer of manufacturing to regions where labor cost is lower or are closer to distribution areas. Also, rational microbicide product demand and usage forecasting modeling, as well as the use of already relevant commercial (*e.g.*, condoms), health systems or civil society organizations distribution channels may be advantageous (229). Finally, products potentially providing long-term protection, such as rings in the case of vaginal delivery, may also allow savings (cost per dose) despite higher production costs per unit. However, no such rectal microbicide product is currently being developed.

2.4.6. Novel rectal microbicide drug delivery approaches

As the field of rectal microbicides is growing, new ideas are flourishing in order to develop enhanced drug delivery solutions. As described above, rectal microbicides have been mostly developed as aqueous-based formulations, namely hydrophilic

gels. Despite that, non-aqueous systems may also provide interesting platforms for microbicide delivery. For instances, Wang *et al*. (188) proposed two different lipid-based rectal placebo formulations: one liquid (isopropyl myristate, myristyl miristate) and one gel (glyceryl stearate and PEG-75 stearate, caprylic/capric triglyceride, vitamin E acetate). Both were shown non-toxic in human rectal explants and non-irritating upon delivery in a rabbit model, thus potentially providing alternative safe options for rectal microbicide delivery. Further, both systems were prepared under cGMP and could be easily scaled-up to industrial manufacturing.

The use nanotechnology-based drug systems is an emergent strategy for the delivery of microbicide drug candidates, offering several potential advantages over more conventional systems (230). Again, work conducted so far has mainly focused on vaginal microbicides but first steps have already been taken towards the development of nanocarrier-based rectal microbicides (231). Most data obtained so far concerns Vivagel® (Starpharma Pty Ltd.), a carbomer-based gel containing a dendrimer (SPL7013) (232). SPL7013 is not a drug nanocarrier but possesses itself antiviral activity due to the terminal naphthalene disulfonate groups of this fourth-generation dendrimer. Relevant to its potential rectal use, a 5% SPL7013 gel showed reduced toxicity to Caco-2 cells (233), but caused epithelial shedding of colorectal explants despite reducing HIV-1 infection by over 85% (50). Toxicity was associated to the vehicle rather than the dendrimer. A 3% SPL7013 gel was also found safe when tested in pigtailed macaques after trice rectal application for four days (51).

More recently, das Neves *et al*. (47, 234) showed that poly(ethylene oxide)-modified poly(ε-caprolactone)-based NPs (PEO-PCL NPs) could be useful as a potential nanocarrier (200 nm) for the non-nucleoside reverse transcriptase inhibitor (NNRTI) drug dapivirine, namely for rectal administration. Dapivirine-loaded PEO-PCL NPs were able to increase intracellular levels of the drug in Caco-2 cells and different immune cells relevant to HIV sexual transmission, while reducing the cytotoxicity of dapivirine in suspension. However, similar sized NPs prepared with different surface modifiers, namely dodecyl sulfate or cetyltrimethylammonium bromide, presented higher toxicity thus opposing their use (234). These observations were correlated with the intrinsic toxicity of these two compounds. Drug-loaded PEO-PCL NPs also allowed increasing tissue levels in pig rectal explants while reducing the amount of permeating dapivirine (47). These results suggest that proposed NPs

may have the potential to enhance local mucosal drug levels but decrease systemic exposure. However, further expanded studies, including *in vivo* assessment, are required in order to fully understand the value of PEO-PCL NPs as carriers for rectal delivery of dapivirine. In another recent and interesting work, Samizadeh *et al.* (235) studied several *in vitro* and *in vivo* features of amprenavir-PEG-Bac7 nano-conjugates (no size reported) in order to attest their potential as a microbicide for preventing rectal HIV transmission. The incorporation of the cell penetrating peptide (CPP) Bac7, as well as the adequate selection of the MW of PEG, were shown essential in allowing maintenance of the native antiretroviral activity of the ARV drug amprenavir. The full nano-conjugate was not tested *in vivo* but preliminary data for fluorescent labeled amprenavir-$PEG_{3.4kDa}$-FITC and Bac7-$PEG_{3.4kDa}$-FITC surrogates suggested that prolonged colorectal retention was possible due to the presence of the CPP. However, more studies with the actual drug nano-conjugate are required in order to fully assess the potential of the system (235).

Another conceptual approach is that of orally-delivered microbicides. This strategy differs from oral pre-exposure prophylaxis (PrEP) in that products are intended to release API(s) in the vicinities of the colorectum in order to allow topical action and minimize systemic absorption. Despite evident technical and physiological challenges, colorectal drug delivery is a well-developed topic and different approaches towards the problem have been proposed (236). This strategy might help circumvent acceptability and adherence issues but, at the same time, its implementation would require long-term use since rapid onset of protection would not be possible with discontinuous use. In an exploratory study, Li *et al*. (237) described commensal bacteria that were genetically-modified in order to secrete cyanovirin-N. After incorporation in yogurt and fed to pigtail macaques, these bacteria were able to deliver cyanovirin-N to the rectal cavity and reduce viral peak levels in rectal biopsies challenged *ex vivo* with SHIV. However, complete inhibition of viral replication was not achieved, thus suggesting that additional measures (*e.g.*, bacteria secreting multiple antiviral agents, refinement of the delivery vehicle) are required. In another report, Zhu *et al*. (238) developed poly(lactide-*co*-glycolide) NPs, loaded with a peptide antigen and toll-like receptor ligands, incorporated in pH-sensitive microparticles based on polymethacrylate copolymers for the induction of colorectal immunity. The NP-in-microparticle system was orally delivered to BALB/c mice and

shown equally effective as the vaccine delivered by the colorectal route in protecting animals from colorectal challenge with vaccinia virus. Again, this study seems to backup the possibility of an oral-delivered, rectal-targeted microbicide approach.

2.5. Conclusions

Despite great advances, the development of rectal microbicides is still in its infancy. Formulation and product design aspects, as well as acceptability issues, are attracting increased interest in the field of HIV prevention due to their tremendous impact on the potential success (or failure) of microbicides. After several years following on the footsteps of vaginal counterparts, rectal microbicides are gaining momentum but concise guiding for the development of such products is still missing. Despite that, current opinion favors formulating specific rectal microbicides in view of the present knowledge on the mechanisms of rectal HIV infection and product interactions with the rectal environment (mucus, content, microbiota, among others). Still, some questions related with the ideal volume required to provide complete protection, suitability of the gels and other dosage forms to incorporate and properly deliver novel microbicide candidates, product distribution and retention along mucosal surfaces and at target sites, and user preferences for specific dosage forms remain to be answer. Overall, one rectal microbicide product may not fit all and the development of various equivalent products may be an interesting approach. Also, dual compartment products are tempting and are being actively explored, but their feasibility still needs to be clearly demonstrated. Finally, recent trend towards the development of next-generation combination microbicides is an important aspect of the field, which poses substantial challenges to formulation scientists. Presently, a standard safe, effective and highly acceptable rectal microbicide still seems elusive but, as explored in this work, may be achievable. Innovative drug delivery approaches and novel strategies are also now emerging and, by their own or allied to conventional dosage forms, may provide highly sophisticated delivery solutions for rectal microbicides.

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CHAPTER 3

Surface modification with polyethylene glycol enhances colorectal distribution and retention of nanoparticles

The information provided in this chapter was based in the following publication:

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3.1. Abstract

Dense surface modification with short chain polyethylene glycol (PEG) has been previously demonstrated as favoring the transport of nanoparticles (NPs) across mucus. However, the ability of such approach to influence the distribution and retention of NPs along the length of the colorectum after rectal delivery has not been previously established. Herein, the distribution and retention of poly(lactic-*co*-glycolic acid) (PLGA) NPs modified with PEG in a non-covalent fashion are reckoned in a mouse model. Despite overall rapid depletion, both PEG-modified and non-modified NPs are able to reach the middle segment of the colon. PEG-modified NPs are able to enhance retention up to at least two hours post-administration, contrasting with nearly residual levels observed for non-modified NPs after 15 minutes. The ability of PEG-modified NPs to putatively cross mucus also appears to promote association with tissues. Overall, the work provides significant insights as to the behavior of NPs in the colorectum, which could be valuable for the development of rectal nanomedicines. It further reinforces the potential usefulness of PEG-modified NPs as mucus-penetrating carriers for mucosal drug delivery.

3.2. Introduction

Rectal administration of drugs is a long and well-established medical practice. Its value has been demonstrated for both the management of local conditions (*e.g.*, inflammatory bowel disease (1), irritable bowel syndrome (2) or constipation(3)), and systemic diseases, particularly in children and the elderly (4, 5). Also, in recent years, advances in rectal drug delivery have been mostly set by efforts in developing anti-HIV microbicides (6). The rectal route typically provides the possibility to increase drug levels at mucosal fluids and tissues that could not be otherwise safely obtained by oral or intravenous administration. Various products for rectal use are available in the form of enemas, suppositories, foams or semisolid systems (*e.g.*, gels). However, conventional dosage forms frequently display disadvantageous features such as poor colorectal distribution and/or retention, thus leading to sub-optimal PK (7).

Nanotechnology-based drug delivery systems may help circumventing these problems depending on adequate engineering. One particular aspect determining the fate of drug carriers is that of the interaction with mucus. Despite commonly considered as beneficial, mucoadhesive properties of nanosystems lead to their reduced ability to be transported in mucus and cross this barrier towards the mucosal surface (8). The development of mucus penetrating NPs by densely modifying the surface of otherwise mucoadhesive systems with linear, low MW PEG has been a major breakthrough in order to potentially promote distribution and retention at mucosal sites (9-11). PEG provides an inert surface layer that allows abbreviating the establishment of adhesive interactions with mucin chains. In particular, extensive *in vivo* data support that PEG-modified NPs enhance coating of the vaginal mucosa by NPs while increasing retention *in loco* (12-15). Similar behavior has been described for NPs at both the lower and upper intestinal tract; distribution of mucoadhesive systems was generally restricted to the jejunal/colorectal lumen whereas mucus penetrating NPs featured improved coating of epithelial surfaces (16, 17).

However, several questions regarding the advantages of mucus penetrating NPs remain unclear. For instance, most *in vivo* studies so far conducted have only presented results regarding qualitative distribution of NPs across discrete sections of

mucosae, highlighting lumen-epithelial transport. Moreover, no data are available regarding their time-dependent and longitudinal distribution (*i.e.*, along the full extension of the colon) as compared to mucoadhesive counterparts upon administration in the rectum. Understanding the distribution and retention dynamics of NPs throughout the different parts of the colorectum over time, both qualitatively and quantitatively, may further support the value of nanotechnology-based carriers for rectal administration of drugs. Moreover, such information may help settling the ongoing debate on whether mucus penetrating NPs may provide or not significant advantage over mucoadhesive ones, in particular for the rectal route (18).

Herein, we produced and extensively characterized putative mucus penetrating PLGA NPs by densely modifying their surface with PEG of approximately 5 kDa in a non-covalent way using poloxamer 407, a triblock copolymer of PEG-PPG-PEG. These NPs were then tested in a mouse model for quantitative distribution throughout the length of the colorectum after rectal administration. Generated data indicated that mucus penetrating NPs performed better than non-modified mucoadhesive counterparts in terms of *in vivo* distribution and retention.

3.3. Materials and Methods

3.3.1. Materials and animals

End-capped PLGA with 50:50 D,L-lactide:glycolide ratio and 0.2 dL g^{-1} inherent viscosity (Purasorb PDLG 5004) was a kind offer from Corbion (Gorinchem, The Netherlands). Poloxamer 407 (Kolliphor® P 407) was purchased from BASF (Ludwigshafen, Germany), Coumarin-6 (C6) from Sigma-Aldrich (Schnelldorf, Germany) and Cyanine7.5 carboxylic acid (Cy7.5) from Lumiprobe (Hannover, Germany). All other materials and reagents were of analytical grade or equivalent. Male CD-1 IGS mice were acquired from Harlan (Barcelona, Spain). All experiments were approved by the Ethics Committee at Instituto Universitário de Ciências da Saúde (process no. 01/ORBEACESPU/2014) and conducted at CESPU and i3S animal facilities under FELASA and the European Directive 2010/63/EU guidance.

3.3.2. Production of nanoparticles

PLGA NPs were produced by a nanoprecipitation method (19). Twenty milligrams of polymer were dissolved in acetone (1 mL) and slowly injected using a 23G needle into a poloxamer 407 aqueous solution (0.1% w/v) under magnetic stirring (**Figure 3.1**). NPs were collected after 3 h under stirring at room temperature, washed twice with 10 mL of ultrapure water using a 100 kDa MWCO filter system (Amicon® Ultra-15 filter, Tullagreen, Ireland) and concentrated to 20 mg mL^{-1}. Particles (20 mg) were modified with PEG by incubation into 9 mL of poloxamer 407 aqueous solutions of different concentrations (0% to 4% w/v) for 12 h. Plain PLGA NPs (incubated in water without poloxamer 407) and PEG-modified counterparts (PEG-PLGA NPs) were again concentrated to 20 mg mL^{-1} using Amicon® filters. NPs were freeze-dried whenever required. Fluorescent PLGA NPs and PEG-PLGA NPs were obtained using the same methodology but co-dissolving either 0.8 mg of C6 or 4 μg of Cy7.5 with PLGA in acetone. The fluorescent signal intensity of PLGA NPs and PEG-PLGA NPs was equivalent for each probe.

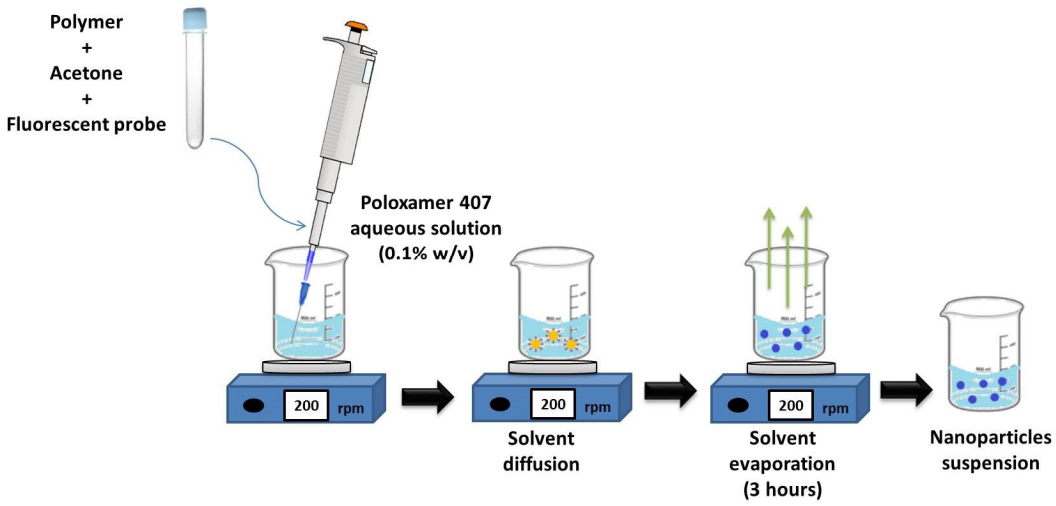

Figure 3.1. Schematic representation of the used nanoprecipitation technique.

3.3.3. Characterization of nanoparticles

NPs were characterized for hydrodynamic diameter and polydispersity index (PdI) by dynamic light scattering (DLS), and zeta potential (ZP) by laser Doppler anemometry (LDA) after dispersion at a concentration of 0.2 mg mL^{-1} in 10 mM sodium chloride solution (pH 7.0) using a Zetasizer Nano ZS (Malvern, Worcestershire, UK) at 25˚C. Surface morphology and size confirmation of NPs was performed by transmission electron microscopy (TEM) using a JEM-1400 microscope (JEOL, Tokyo, Japan) at an acceleration voltage of 80 kV. Ten microliters of the dispersion of NPs (20 mg mL^{-1}) were placed on a grid and treated with uranyl acetate before imaging. Energy dispersive spectroscopy (EDS) elementary analysis was performed by mounting samples of NPs on nickel grids and a beryllium holder (EM-21150, JEOL) and using an X-Max 80 mm^2 (Oxford Instruments, Bucks, England) operated at 80 kV coupled to the TEM. Chemical characterization of the surface of freeze-dried NPs was further conducted by proton nuclear magnetic resonance (^1H NMR) using a Bruker Avance III 400 Novo (Billerica, MA, United States). Spectra of NPs dissolved in dimethyl sulfoxide-d_6 (DMSO-d_6) were obtained at room temperature, 400 MHz, 10 s relaxation time, and 90˚ pulse. Briefly, a known amount of PEG-PLGA NPs, PLGA NPs and poloxamer 407 was weighted and dissolved in DMSO-d_6. Quantification of PEG was performed by integration of corresponding peak areas. Contact angle measurements of NP-coated surfaces were conducted by using the sessile drop method in an OCA 15 measuring device (DataPhysics, Filderstadt, Germany) equipped with a CCD video camera and SCA 20 software as previously described (20). Briefly, gold-coated silicon wafers (INESC-MN, Lisbon, Portugal) were homogenously covered with approximately 50 μL of aqueous suspensions of NPs (20 mg mL^{-1}) and left to dry overnight at room temperature under normal pressure, followed by 1 h under vacuum. Surface images taken at 2 s intervals over a total of 200 s were used to determine the droplet profiles of 4 μL of ultrapure water. Profiles were fitted using the ellipse method and water contact angle values were calculated by extrapolating the time-dependent curve to zero. The stability of PEG coating was determined by placing NPs suspended in water (0.5 mg mL^{-1}) at 37˚C

under 100 rpm magnetic stirring. Samples were collected over 120 h, diluted to 0.2 mg mL^{-1} with sodium chloride solution (final concentration of 10 mM, pH 7.0), and the ZP measured by LDA.

3.3.4. *In vivo* studies

Mice (7-12 week old) were fasted for 24 h with free access to water before experiments. The rectum was also gently flushed with 200 µL of water using a plastic pipette tip, 30 min prior to the administration of NPs. Fresh fluorescent (C6- or Cy7.5-loaded) PLGA NPs or PEG-PLGA NPs (0.9 mg) were dispersed in 20 µL of PBS, pH 7.4 and administered with the aid of a plastic pipette tip inserted in the colorectum to a depth of approximately 0.5 cm. Mice were restrained in a head-down position for approximately two minutes in order to minimize leakage before returning to individual cages under unrestrained conditions and re-allowed access to food and water. Whole body near infrared (NIR) imaging of animals treated with Cy7.5-loaded NPs was performed at pre-established time points by using an IVIS Lumina LT system (Perkin Elmer, Waltham, MA, USA) after brief anesthetization with inhalational isoflurane. Abdominal fur was removed with a hair clipper before imaging. Background signal from non-treated animals was used to establish relevant radiance signal ($\geq 0.4\times10^8$ p cm^2 s^{-1} μW^{-1}).

Animals were sacrificed at 15 min, 2 h or 6 h by isoflurane overdose followed by cervical dislocation. Necropsy was then performed and the terminal fraction of the GIT isolated. In the case of animals administered with Cy7.5-loaded NPs, tissues were placed in petri dishes and analyzed using the IVIS system. Background signal from the terminal GIT of non-treated animals was used to establish the relevant radiance signal ($\geq 0.6\times10^8$ p cm^2 s^{-1} μW^{-1}). Quantification of the radiant efficiency from same sized regions including complete excised tissues was performed using Living Image® software v. 4.4 (Caliper, Hopkinton, MA, USA). Grey-scale photographs superimposed with radiance signal maps were used to manually determine the maximum distance covered by NPs, expressed as the maximum percentage of the full length of the colorectum, with the aid of the segmented line tool

from ImageJ software (v. 1.51j8, NIH, Rockville, MD, USA). As for mice treated with C6-loaded NPs, the terminal part of the GIT was divided into three equal sized segments: distal (included the rectum and anus), middle and proximal. Cecum and the terminal portion of the ileum (roughly 2 cm) were also collected for analysis. The central portion of each segment (corresponding to approximately 5 mm) was collected, frozen at −80 ˚C in O.C.T. Compound (Thermo Scientific, Runcorn, UK) and processed for fluorescence imaging. Briefly, 7 μm transversal cryosections were stained by DAPI (4',6-diamidino-2-phenylindole dihydrochloride) (Sigma-Aldrich) and CellMask™ Orange Plasma membrane Stain (Thermo Scientific), and mounted using Vectashield® (Vector Laboratories, Burlingame, CA, USA). Images were obtained using an Axiovert 200M inverted fluorescent microscope (Carl Zeiss, Göttingen, Germany). In the case of quantitative analysis, each colon segment was flushed with PBS (5×50 μL) and collected fluids, as well as tissues, were stored at −80 ˚C until further processing. After thawing, washing fluids and tissues were mixed with acetonitrile, homogenized either by using a vortex for fluids or an Ultra-Turrax processor (IKA®-Werke, Staufen, Germany) for tissues, resulting homogenates centrifuged at 13,414 ×*g* for 10 min at 4˚C, and the supernatants collect and used for assaying at 460/540 nm. C6 standards were similarly prepared and used for generating calibration curves. The method was linear over the range of 0.02-5 μg mL^{-1} ($R^2 \geq 0.9987$), with detection and quantification limits of 0.01 μg mL^{-1} and 0.02 μg mL^{-1}, respectively, as assessed based on the standard deviation (SD) of the response and the slope (21). C6 fluorescence quantification in samples was conducted by using a Synergy™ Mx microplate reader (BioTek Instruments, Winooski, VT, USA).

3.3.5. Statistical analysis

Analysis was performed using Student's *t*-test (two group comparisons) or one-way ANOVA with post-hoc Tukey's HSD test (multiple comparisons). All data were processed using Prism 5.01 software (GraphPad Software, La Jolla, CA, USA) and $p < 0.05$ was accepted as denoting significance.

3.4. Results and Discussion

3.4.1. Production and characterization of nanoparticles

We successfully produced PLGA NPs by adapting a previously established method (19). NPs featured mean values for hydrodynamic diameter, PdI and ZP of 221 ± 1 nm, 0.066 ± 0.022 and −6.6 ± 0.2 mV, respectively. Further, non-covalent coating of NPs with PEG (PEG-PLGA NPs) was pursued by incubation with a poloxamer 407 solution in order to allow surface adsorption. The molecular mass ratio of PEG (4.5×10^3 g mol^{-1}) and PPG (3.3×10^3 g mol^{-1}) in the selected triblock copolymer has been previously shown ideal to provide dense surface coverage of NPs and enable mucus diffusive properties (10). Increasing concentrations of the poloxamer 407 solution were first tested in order to optimize the best conditions for PEG modification. Colloidal features of NPs did not change significantly when incubated in up to 4% (w/v) poloxamer 407, as compared to PLGA NPs, except for ZP (**Fig. 3.2**). Non-coated NPs presented mildly negative ZP values around −7 mV, while near neutral ZP was observed for PEG-modified NPs. Increasing poloxamer 407 concentrations starting at 0.5% (w/v) did not modify significantly ZP, although a trend for increasing values was still apparent between 0.5% and 1%. This last concentration was thus selected for further studies in order to potentially assure dense PEG coating of the surface of NPs (22). Higher concentrations of poloxamer 407 were not considered, as saturation of PEG at NP surface appears to be achieved (**Fig. 3.2**). The physicochemical characteristics of the final nanosystems are presented in **Table 3.1**.

PLGA NPs and PEG-PLGA NPs were also characterized by TEM, which confirmed size values obtained by DLS measurements of typically round shaped and smooth surfaced particles (**Fig.3.3**). Importantly, auxiliary experiments comprising EDS, ^{1}H NMR and contact angle analysis further confirmed the presence of PEG at the surface of PEG-PLGA NPs as compared to PLGA NPs. In particular, increased carbon and decreased oxygen contents obtained for PEG-PLGA NPs as compared to non-modified particles in EDS analysis suggests the higher content in PEG

[$H(OCH_2CH_2)_nOH$] and shielding of the PLGA core [$(C_3H_4O_2)_x(C_2H_2O_2)_y$] (23) (**Table 3.2**).

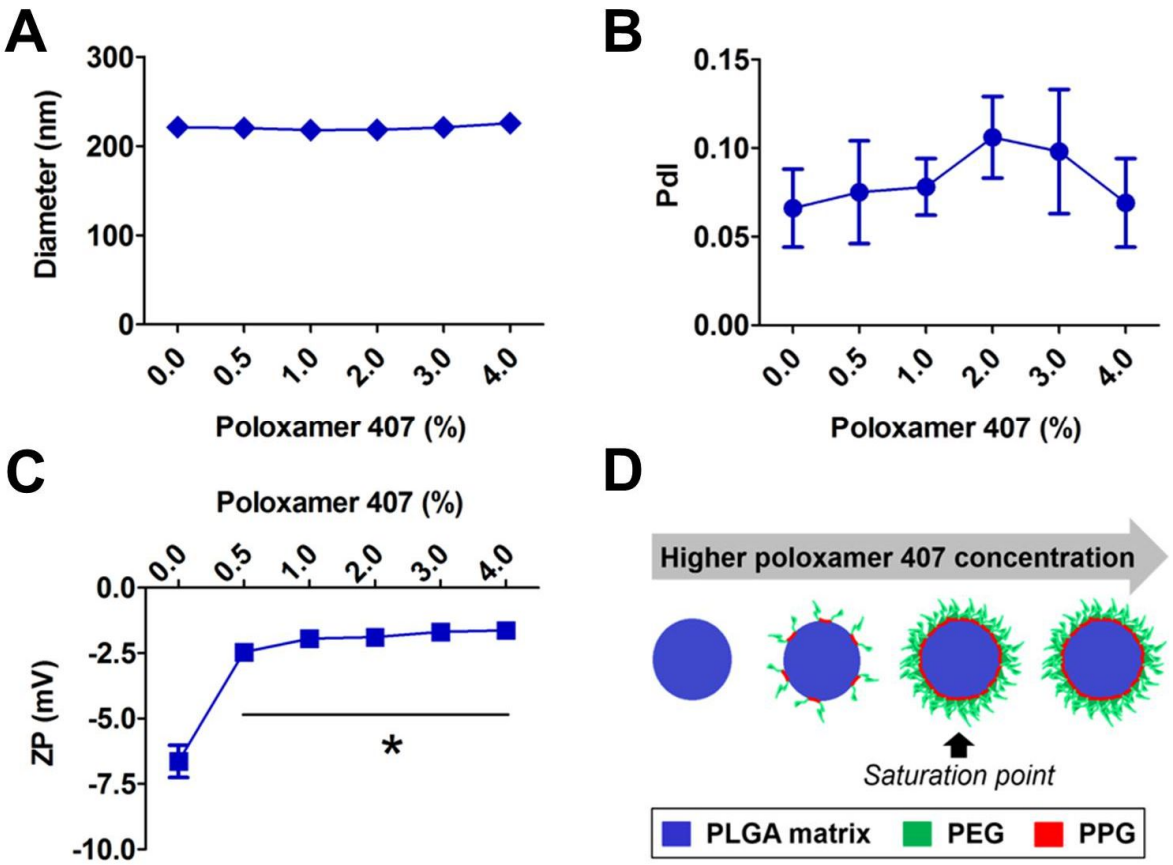

Figure 3.2. Colloidal features of NPs. Changes in (**A**) hydrodynamic diameter, (**B**) polydispersity index (PdI) and (**C**) zeta potential (ZP) values of PLGA NPs incubated with increasing concentrations of poloxamer 407. Results are presented as mean ± SD (n=3). (*) denotes a significant difference (p < 0.05) from NPs produced without incubation with poloxamer 407 (0.0%). (**D**) Graphical depiction of increasing NP surface density and saturation of PEG coating.

Table 3.1. Physicochemical features of PLGA and PEG-PLGA NPs. Hydrodynamic diameter (HD), polydispersity index (PdI), zeta potential (ZP), association efficiency (AE) and drug loading (DL) of empty and C6-loaded PLGA and PEG-PLGA NPs. Results are presented as mean ± SD (*n*=3).

Nanosystem	Payload	HD (nm)	PdI	ZP (mV)	AE (%)	DL (%)
PLGA NPs	--	221 ± 1	0.066 ± 0.022	-6.6 ± 0.6	--	
	C6	190 ± 3	0.332 ± 0.078	-13.1 ± 1.1	99.9 ± 0.0	3.8 ± 0.0
PEG-PLGA NPs	--	218 ± 4	0.078 ± 0.016	-1.9 ± 0.1	--	
	C6	191 ± 6	0.173 ± 0.058	-3.8 ± 1.6	99.8 ± 0.1	3.8± 0.0

Table 3.2. Energy dispersive spectroscopy results of NPs. Results denote the presence of carbon and oxygen at the surface of PLGA NPs and PEG-PLGA NPs. Results are presented as mean ± SD (*n*=3).

Samples	% C	% O
PLGA NPs	93.7 ± 0.3	5.6 ± 0.2
PEG-PLGA NPs	97.5 ± 0.1	2.5 ± 0.1

^{1}H NMR analysis corroborated the results obtained by LDA and EDS analysis regarding the surface coating of NPs surface with poloxamer 407. Representative ^{1}H NMR spectra of PLGA NPs, PEG-PLGA NPs and poloxamer 407 are presented in **Figure 3.4**. All spectra data were calibrated through the solvent peak at 2.5 ppm and all of them showed a characteristic peak at 3.3 ppm attributed to the presence of water (24). Both types of NPs presented typical peaks of PLGA at 1.47, 4.8 and 5.2 ppm that are related with the presence of $-CH_3$, $-CH_2$, and $-CH$ protons, respectively (25). Appearance of peaks at 1.04 ppm ($-CH_3$) and between 3.4 and 3.6 ppm

(attributed to –CH and $-CH_2$) suggested the presence of poloxamer 407 (26). In the case of PLGA NPs, the presence of poloxamer 407 was residual and resulted from the use of this polymer as stabilizer during the production of NPs. Integration and normalization – required due to different MW and monomer repetition – of typical peaks of poloxamer 407 (1.04 ppm) and PLGA (1.47 ppm), showed a shift in PLGA:poloxamer 407 ratio from around 8 to 3, thus suggesting surface modification of NPs with PEG.

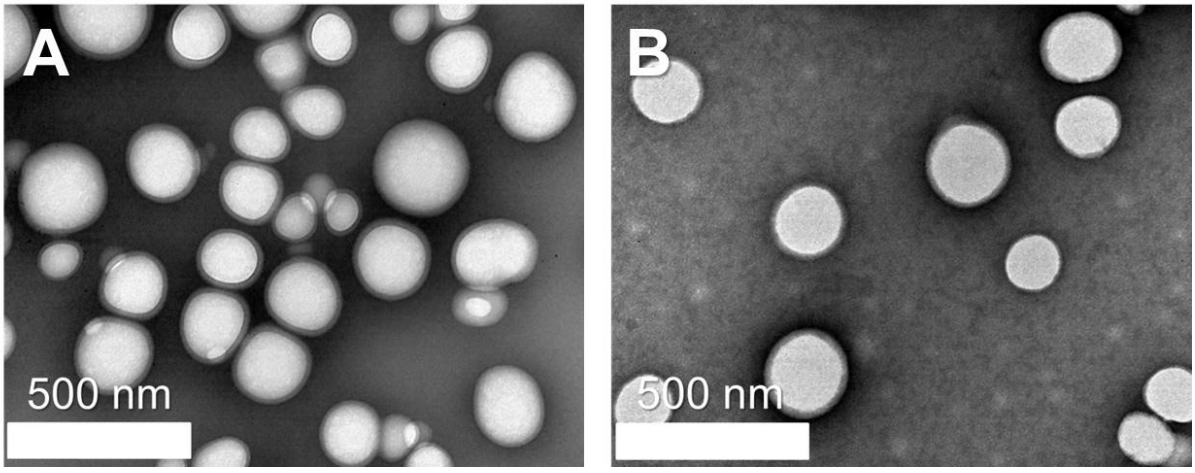

Figure 3.3. Transmission electron microscopy analysis of NPs. Representative images of (**A**) PLGA NPs and (**B**) PEG-PLGA NPs. Both type of NPs presented round shape and smooth surface. Results are in agreement with data obtained by dynamic light scattering (DLS) measurements regarding diameter and size distribution.

Further, contact angle measurements were performed in order to assess the hydrophobicity-hydrophilicity profile of both types of NPs. Contact angle value of PEG-PLGA NPs decreased around 10˚ compared to contact angle value of PLGA NPs (53.3˚ Vs 62.0˚), corroborating the higher hydrophilicity of PEG-PLGA NPs, consistent with the presence of hydrophilic PEG chains at NPs surface (27) (**Figure 3.5**).

Despite their non-covalent nature, PEG adsorption to NPs was also shown persistent for at least 24 h when submitted to an *in vitro* washing protocol (**Fig. 3.6**).

Values for ZP were kept relatively constant within the range of −2 to −3 mV up to 24 h. A slight but significant decrease was observed only at 72 h and 120 h. These results are in line with previous work that evidenced strong and stable adsorption of poloxamer 407 to PLGA NPs for at least 24 h (10). Taken together, these data support the prolonged and dense non-covalent coating of PLGA NPs with PEG, and substantiate their ability to be used as mucus penetrating NPs for subsequent *in vivo* studies (10).

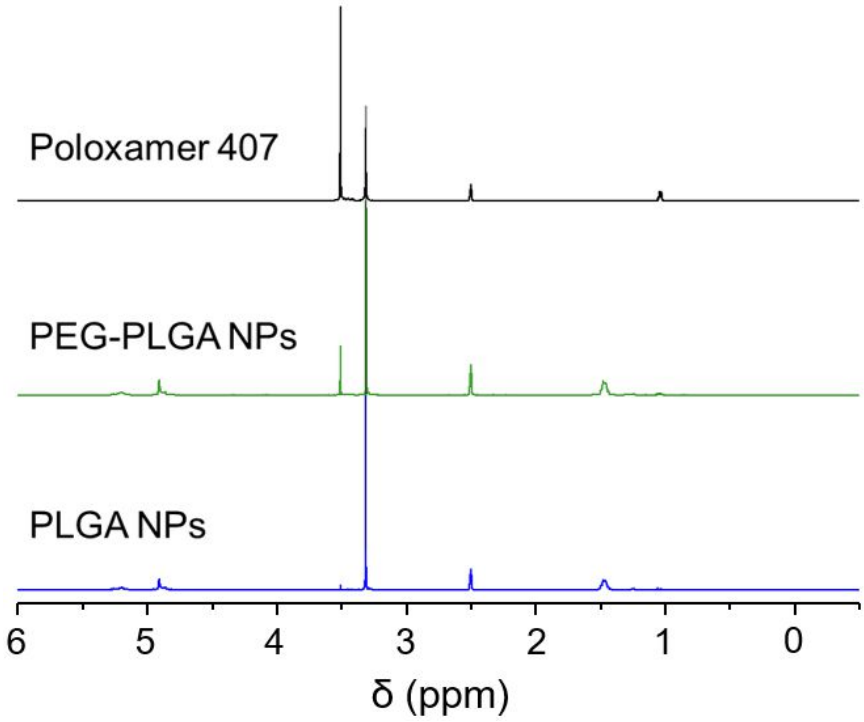

Figure 3.4. ^{1}H NMR analysis of NPs. Representative spectra of PLGA NPs, PEG-PLGA NPs and Poloxamer were obtained in DMSO-d6 at 400 MHz (calibration performed with the solvent peak at 2.5 ppm).

C6 was further incorporated into NPs in order to render fluorescent signal. Colloidal features of both C6-loaded PLGA NPs and C6-loaded PEG-PLGA NPs were similar to non-fluorescent counterparts (**Table 3.1**). Moreover, the release of C6 from NPs was low under *in vitro* conditions (less than 9% in PBS, pH 7.4 at 24 h)

thus assuring general co-localization of C6 fluorescent signal and NPs during animal studies (**Figure 3.7**).

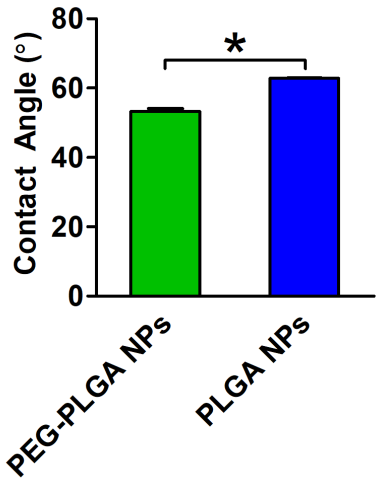

Figure 3.5. Contact angle values of NPs. Water optical contact angle measurements of gold surfaces coated with PLGA NPs or PEG-PLGA NPs. Data analysis sustains a significant decrease in contact angle from mean 62.0 to 53.3 degrees when NPs were modified with poloxamer 407, which is consistent with the presence of hydrophilic PEG chains at the surface of NPs (28). Results are expressed as mean ± SD (*n*=3). (*) denotes a significant difference ($p < 0.05$).

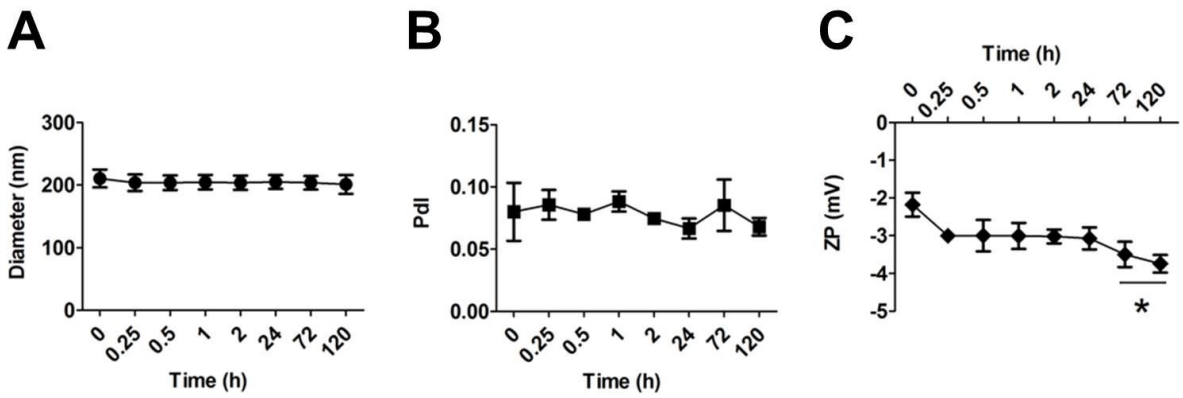

Fig. 3.6. Persistence of PEG coating of NPs. Data represents changes in (**A**) hydrodynamic diameter, (**B**) polydispersity index (PdI) and (**C**) zeta potential (ZP) of PEG-PLGA NPs over 120 h when in water (37 ˚C, 100 rpm magnetic stirring). No changes in size and size distribution were apparent throughout the experiments. Results are presented as mean ± SD (*n*=3), except for 120 h (*n*=2). (*) denote statistically significant differences ($p < 0.05$) from NPs at time zero.

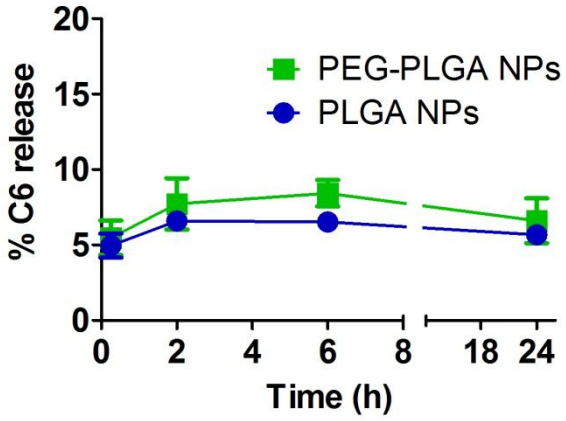

Figure 3.7. *In vitro* release profile of C6 from PLGA and PEG-PLGA NPs. Release of C6 was assessed over 24 hours in PBS pH 7.4 under sink conditions. Each point represents the mean values and bars the SD (*n*=3).

3.4.2. *In vivo* distribution and retention of nanoparticles

We then proceeded with *in vivo* studies concerning the distribution of fluorescent NPs using a murine model. Male CD-1 IGS mice were fasted for 24 h and had their distal colon/rectum washed with water before the administration of NPs in order to soften and minimize fecal content and output, thus better resembling the human colorectal content (16). Animals were further allowed to rest for 30-40 min in order to replenish the normal mucus layer (29). Fluorescent PLGA NPs or PEG-PLGA NPs (0.9 mg) were suspended in PBS and instilled intrarectally. Animals were conscious throughout all procedures and left unrestrained until being euthanized at pre-determined time points (15 min, 2 h and 6 h). We began by observing the behavior of PLGA NPs or PEG-PLGA NPs in different segments of the colon (proximal, middle and distal) regarding transport from the lumen towards the epithelium. Fluorescent microscopy of excised colon (**Fig. 3.8**) confirmed previous studies reporting that PEG-PLGA NPs are able to migrate across mucus and provide extensive coating of the intestinal epithelium (16, 17, 30). As for PLGA NPs, localization was mostly restricted to the lumen and only apparent at the shortest time point tested. This typical behavior was only observed in the middle and distal

segments of the colon for both types of NPs, since only residual fluorescence signaling was detected at the upper part of the colon. Moreover, at 2 h post-administration, the presence of PEG-PLGA NPs at middle and distal segments was still evident but not in the case of PLGA NPs. Fading of most of the C6 fluorescent signal down to residual levels was clear at 6 h post-administration for both types of NPs. Interestingly, epithelial tissue penetration of mucus diffusive NPs was distinctively noted, particularly at 2 h after administration (**Fig. 3.8**). Overall, these data seem to indicate that PEG-modification may enhance not only the distribution of NPs throughout the colorectal mucosa but also prolong their residence time, as inferred from previous work by Maisel *et al*. (16, 30).

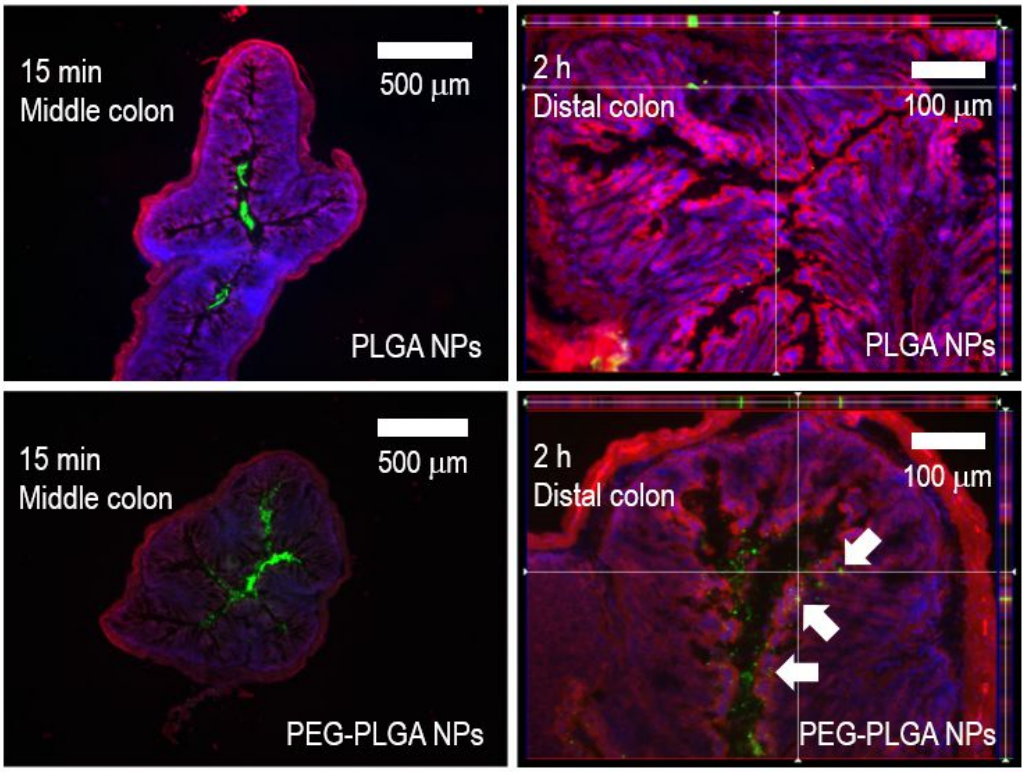

Fig. 3.8. Distribution of NPs in the colon. Representative fluorescent microscopy images of transversal sections in different segments of the colon at 15 min (left panels) and 2 h (right panels) after rectal administration of fluorescent C6-loaded PLGA NPs and C6-loaded PEG-PLGA NPs (see embedded captions for details). Green, blue and red signals are from C6 (associated to NPs), DAPI (DNA) and CellMask™ Orange Plasma membrane Stain (cell membrane), respectively. White arrows highlight NPs embedded into tissue.

In order to better understand the trafficking of both types of NPs along the full length of the colorectum, we conducted studies regarding the quantification of C6 fluorescent signal recovered from different sections of the lower end of the GIT. The terminal part of the ileum, the cecum and colorectum were collected, and individual lavages and tissue homogenates were obtained and processed for fluorescence assessment, as detailed in the experimental section. Distribution of NPs was restricted to the colorectum since no C6 fluorescence signal was observed at the cecum and ileum. Strikingly, the major fraction of administered NPs (above 85%) was lost within 15 min of instillation (**Fig. 3.9, A**). This is most certainly associated with the continuous, intense and unavoidable colonic propulsion and fecal output, typical of mice (31). Although we tried to minimize defecation before administering NPs, fasting and colorectal washing were only partially effective. Adding to the previous, natural turnover and clearance of mucus is likely to also be implicated to some extent in the rapid depletion of NPs (29). Observable leakage and self-grooming, although possible, have not been noted upon inspection throughout the duration of the study and necropsy. Since no previous quantitative studies regarding the retention of NPs following rectal administration have been conducted, it is not possible to have a comparative assessment of the apparently fast depletion of NPs. However, presented results are in line with those for NPs (≈180-270 nm) after vaginal administration to female mice (32, 33).

PEG-PLGA NPs presented overall higher retention in the colorectum as compared to non-modified NPs (**Fig. 3.9**). Differences were only observed at earlier time points with mean 5.5- and 10.5-fold higher amounts of PEG-PLGA NPs at 15 min and 2 h, respectively, as compared to PLGA NPs. No apparent differences were observed at 6 h post-administration. Interestingly, scanty levels at this last point seem to have been reached within a few minutes for PLGA NPs, while relatively stable amounts of PEG-PLGA NPs were achieved after initial clearance and maintained up to at least 2 h. An overall similar trend was observed when considering particles recovered only from the colorectal lumen (lavages) or tissues, as shown in **Fig. 3.9, B-C**. Significant differences were observed at 15 min for tissues and at 2 h for lavages. Although not reaching statistical significance, mean recovery of NPs at 15 min for lavages and 2 h for tissues was also in the order of 4.6- and 15.6-fold higher, respectively, for PEG-PLGA NPs over PLGA NPs.

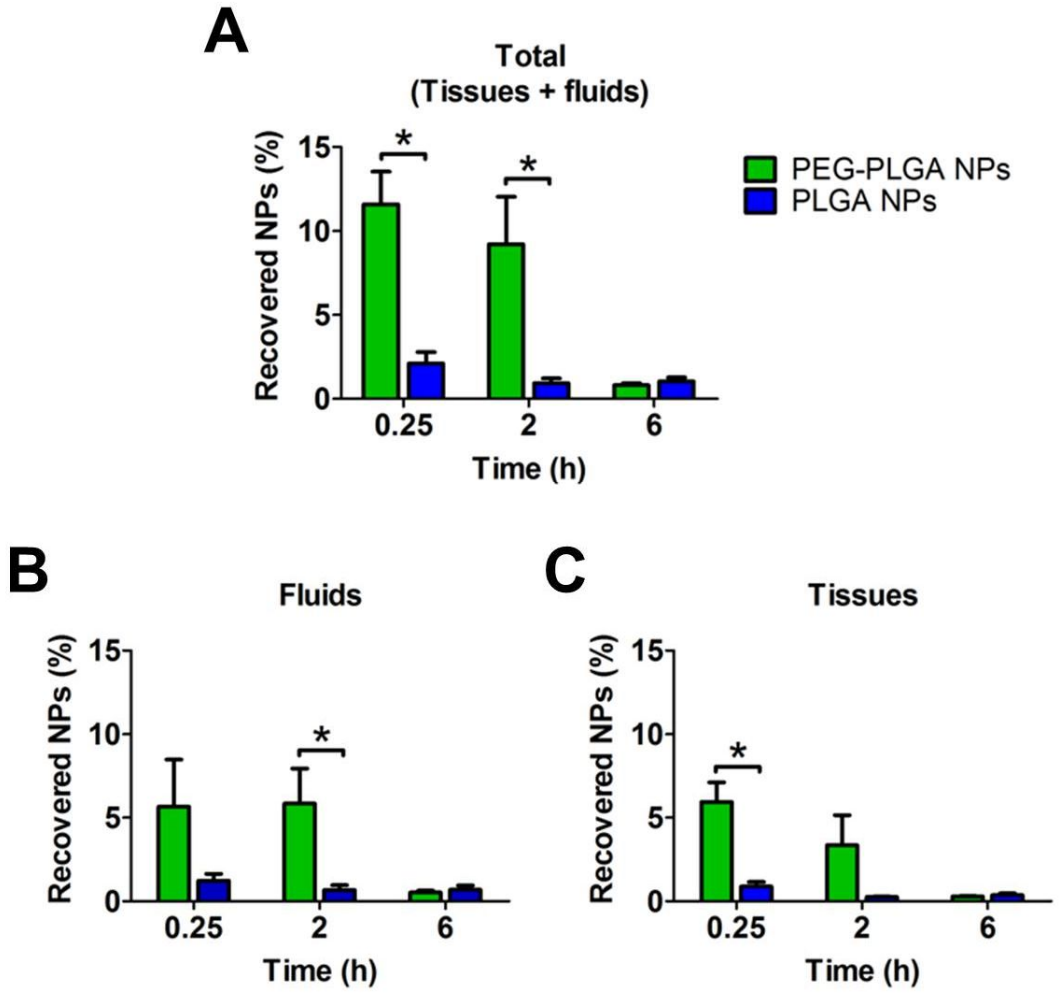

Fig. 3.9. Quantitative distribution of NPs in the colorectum. Recovery of fluorescent C6-loaded PEG-PLGA NPs and C6-loaded PLGA NPs from (**A**) the whole colorectum (tissues and fluids), or only (**B**) fluids and (**C**) tissues at 15 min, 2 h and 6 h following administration. Results are expressed as the percentage of the initial amount of NPs. Columns represent mean values and bars the SD (n=3). (*) denotes a significant difference ($p < 0.05$).

These results confirm previous qualitative studies and suggest that PEG-modification has indeed a decisive impact not only in colorectal retention but also in enhancing tissue uptake. Rapid transport across mucus, particularly at the outermost loosely adherent layers experiencing fast clearance (34), may help decreasing particle depletion and promote interactions and tissue penetration at the mucosa. The enhanced retention and tissue penetration of PEG-PLGA NPs may be of interest for

improving both systemic and local PK, particularly when relevant drug targets are present at the mucosal tissue (*e.g.*, immune cells involved in inflammatory bowel disease pathogenesis (35) or in HIV transmission (36)).

We then proceeded with the analysis of different parts of the colorectum in order to better understand NP distribution throughout this anatomical site following rectal instillation. Three equally sized proximal, middle and distal (including the rectum) segments were considered as schematized in **Fig. 3.10, A**. Again, the presence of both types of NPs was only apparent at the last two thirds of the colorectum, while only residual C6 fluorescent signal was recovered from the proximal segment (**Fig. 3.10, B-C**). Although a precise boundary for distribution could not be assessed, it was clear that NPs could undergo extensive retrograde migration (*i.e.*, against the digestion flow) and reach locations in the colon that distanced several centimeters from the administration site within as little as 15 min. Although the administration of NPs was conducted using a liquid vehicle (20 μL), this was performed in a gentle manner that was unlikely to promote relevant distribution towards the proximal end of the colon.

Curiously, recovery of PEG-PLGA NPs at the distal segment was considerably higher at 2 h as compared to 15 min post-administration, both for lavages and tissues. We hypothesize that, following administration, rapid and intense clearance of NPs at the distal colon/rectum occurred, both to the exterior of the body and, in a retrograde way, towards the middle colon. This may be related to the intense propulsion and trafficking of stiffer fecal pellets at the lower part of the colon, as well as to voluntary defecation movements induced by the presence of fluid at the rectum (37-39). Mucus at the distal segment is also thicker, less permeable to particles and undergoes faster clearance (40, 41), which could further oppose local residence upon administration. Scanty levels of PEG-PLGA NPs observed after 15 min increased up to 2 h, presumably due to the transport of particles that have previously been distributed into the middle segment but are then removed alongside the natural digestion flow. Results for the middle colon also seem to backup this possibility since a decreasing trend was observed starting immediately at 15 min, at which time point maximal amounts of PEG-PLGA NPs were recovered. A similar behavior was not apparent for PLGA NPs, although the constant low levels of fluorescent signal recovered from the distal colon/rectum would likely impair its observation.

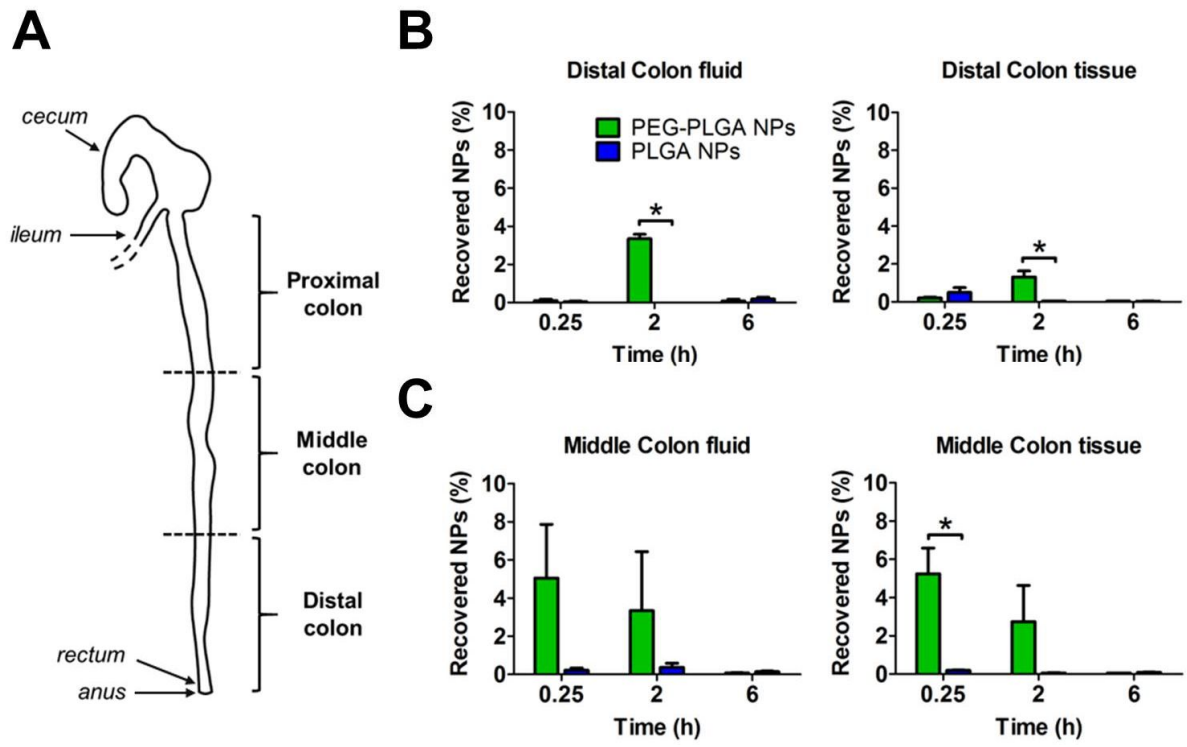

Fig. 3.10. Quantitative distribution of NPs in different segments of the colorectum. (**A**) Schematic representation of the mouse colon and delimitation of segments considered for distribution. Recovery of fluorescent C6-loaded PEG-PLGA NPs and C6-loaded PLGA NPs from fluids and tissues in the (**B**) distal and (**C**) middle sections of the colon at 15 min, 2 h and 6 h following administration. Results are expressed as the percentage of the initial amount of NPs. Columns represent mean values and bars the SD (n=3). (*) denotes a significant difference ($p < 0.05$).

3.4.3. Near infrared imaging of live animals and excised colorectum

To gain more insight into the distribution and retention patterns of different NPs in the colorectum, we proceeded with *in vivo* imaging experiments. For this last purpose, NPs emitting in the NIR were prepared by incorporating Cy7.5 (788/808 nm) rather than C6 in order to minimize tissue scattering and absorbance. Changes in colloidal properties were minimal. Cy7.5-loaded PLGA NPs featured mean hydrodynamic diameter of 210 ± 1 nm, PdI of 0.133 ± 0.015 and ZP of −4.0 ± 0.2 mV, while Cy7.5-loaded PEG-PLGA NPs presented mean hydrodynamic diameter of 218 ± 2 nm, PdI of 0.115 ± 0.009 and ZP of −2.0 ± 0.1 mV. Whole body imaging of mice confirmed fast and extensive leakage after administration of both PLGA NPs and PEG-PLGA NPs, as revealed by the intense fluorescent signal at the perineal region (**Fig. 3.11**). Moreover, mild fluorescence was observed up to 15 min (PLGA NPs) or 1 h (PEG-PLGA NPs) in the abdominal region, suggesting that PEG-modified NPs were able to better retain in the colon (**Fig. 3.11**). Low signal intensity and poor spatial resolution of whole body images were attributed to limited tissue penetration of light and natural folding of the colon that may lead to the presence of NPs at different abdominal depth (42).

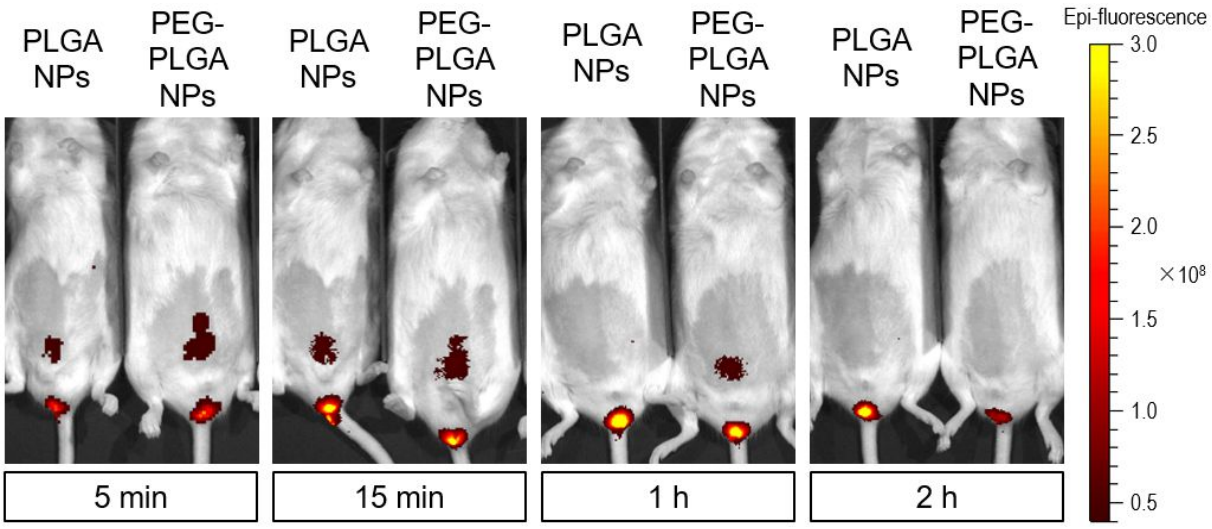

Fig. 3.11. Representative full body imaging of mice after rectal instillation of either Cy7.5-loaded PLGA NPs or Cy7.5-loaded PEG-PLGA NPs. Images were taken at 5 min, 15 min, 1h and 2 h following administration. Heat map scale ranges from 0.4×108 to 3.0×108 p cm2 s−1 μW−1

In order to better estimate the transport and distribution of NPs, colorectal tissues of mice were isolated and analyzed for fluorescence (**Fig. 3.12, A**). Images evidenced that PEG-PLGA NPs were able to migrate farther into the colorectal tract than non-modified NPs. Fluorescent signal was also more intense near the interface between the distal and middle colon at 15 min, thus reinforcing that clearance of PEG-PLGA NPs from the distal segment occurred rapidly following administration, both upwards into the GIT or to the outer body. Complete fading of fluorescent signal was apparent for PLGA NPs at 2 h but still clearly present in the case of PEG-PLGA NPs, namely throughout the middle and distal sections, which seems to agree with quantitative results presented above. Assessment of the maximum distance covered by NPs evidenced that PEG-PLGA NPs were able to be transported across 35-40% of the length of the colon between 15 min and 2 h post-administration (**Fig. 3.12, B**). Coverage by PLGA NPs was restricted to less than 10% at 15 min and negligible after 2 h. Again, these results strongly reinforce the ability of PEG modification to provide enhanced distribution and retention of nanosystems following rectal administration. Semi-quantitative assessment of particle retention was inferred from the total radiance signal from colorectal tissue images (**Fig. 3.12, C**) and was in general agreement with quantitative results previously presented. Differences are likely to be related with the intrinsic variability of tissue scattering and absorption, which limits precise quantitative assessment of fluorescent signal (43, 44).

Several limitations of our study should be considered when interpreting presented results. Naturally, interspecies differences, namely in GIT anatomy and physiology, impair direct transposition of generated data into the human scenario. For example, the overall GIT transit time in mice is much faster (45) and, thus, presented timeframes for distribution and depletion of NPs are probably wider in humans. The use of alternative animal models (*e.g.*, pigs) that are considered closer to humans may be of interest in future studies. Furthermore, mice used in this study were healthy and are not expected to replicate possible variations in colon motility and mucus structure that can occur in models featuring local pathological conditions (46, 47). Other natural changes, namely related with digestion in fed animals, may impact bowel physiology. It is expected, however, that the distribution and retention patterns of NPs reported in the present study may still apply, at least to some extent. PEG modification of particles using a non-covalent approach is not exempt of

criticism since changes throughout the course of the study, namely regarding surface density of poloxamer 407, may have occurred.

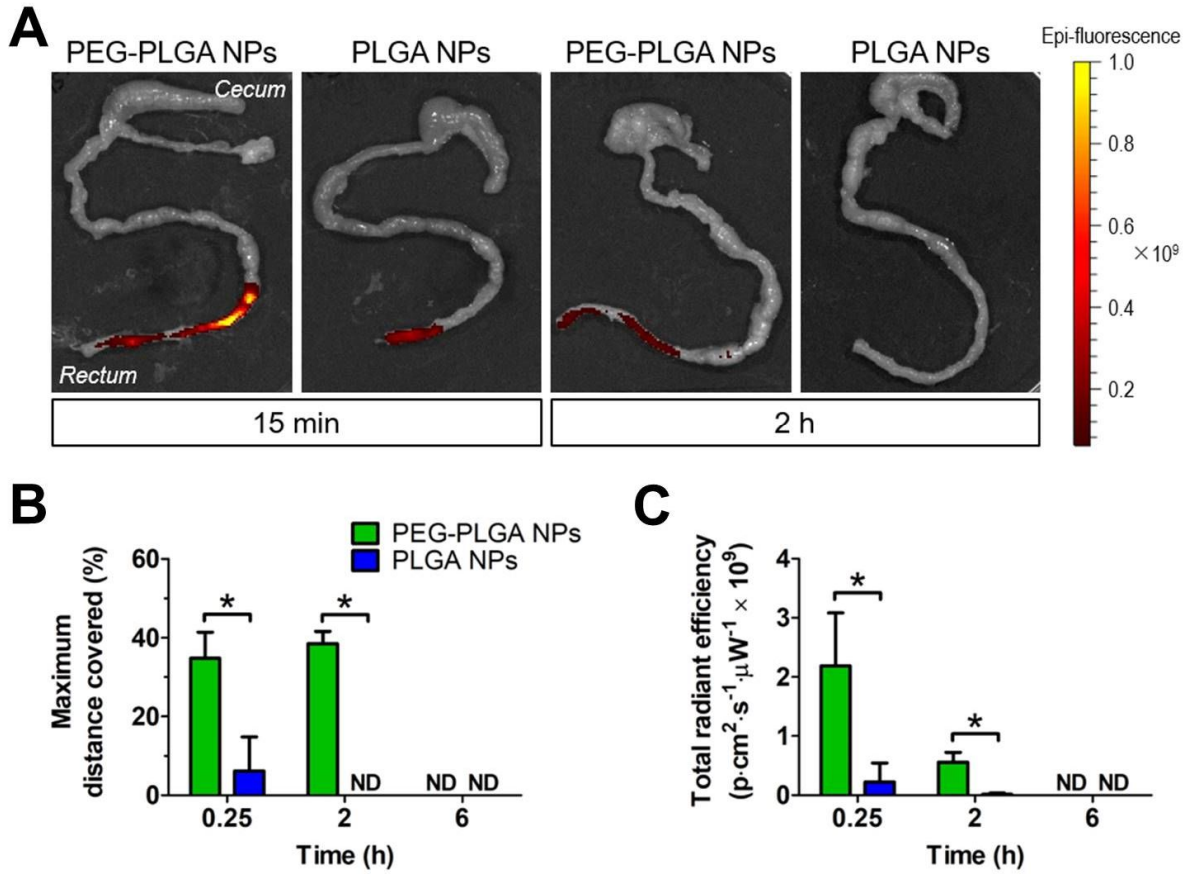

Fig. 3.12. Qualitative and semi-quantitative assessment of the distribution and retention of NPs using NIR imaging. **(A)** Typical fluorescence signal from excised terminal segments of the GIT at 15 min and 2 h following treatment with either Cy7.5-loaded PEG-PLGA NPs or Cy7.5-loaded PLGA NPs. Heat map scale ranges from 0.6×10^{8} to 10×10^{8} p cm^{2} s^{-1} μW^{-1}. **(B)** Estimation of the total extension of the colorectal length covered by NPs. **(C)** Semi-quantitiative evaluation of the amount of NPs associated with excised tissues. Columns represent mean values and bars the SD (n=2-3). (*) denotes a significant difference (p < 0.05). ND: not detected/not determined.

However, *in vitro* data appears to support the considerable resistance of proposed PEG-PLGA NPs to surface shedding of poloxamer 407 (**Figure 3.6**). Moreover, the use of such strategy, namely considering drug delivery applications, may facilitate

regulatory approval as both PLGA and poloxamers (but not PEG-PLGA copolymers) have a long track record of clinical use in drug delivery systems and/or medical devices (48). Another question relates to the relatively broad division of the colon in three parts, as well as the wide time points considered in this study, which limits the precision to which spatial and temporal distribution could be determined. However, considering that this has been a relatively unexplored question, it seems reasonable that the chosen experimental setup can be helpful in establishing a general overview of distribution and retention of NPs upon rectal administration. Also, the inclusion of NIR imaging experiments was able to complement time-dependent distribution patterns of the different NPs.

3.5. Conclusions

We developed and characterized PLGA NPs densely modified at the surface with PEG in a non-covalent way. Materials used for production were selected based on their well-established safety profile and regulatory status for medical use. Both PEG-modified and plain PLGA NPs were shown to reach up to the middle segment of the colon but presented diverse behavior upon rectal administration in a healthy mouse model. In particular, PEG-PLGA NPs featured enhanced distribution and retention as compared to PLGA NPs. These observations were correlated with the ability of modified particles to be transported across mucus and reach the epithelial lining. We also described for the first time an interesting event regarding the distribution dynamics of PEG-PLGA NPs in the distal colon. Early fast depletion of administered particles at the terminal part of the colon to adjacent sites is followed by later replenishing, presumably proceeding from NPs that initially migrated to the middle colon. These circumstances may have implications when considering the use of nanoparticulate drug carriers. Overall, our results reinforce previous reports on the potential usefulness of mucus-penetrating NPs for mucosal drug delivery.

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CHAPTER 4

Non-covalent PEG coating of nanoparticle drug carriers improves the local pharmacokinetics of rectal anti-HIV microbicides

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4.1. Abstract

Antiretroviral drug nanocarriers hold great promise in the development of microbicides to be used in the prevention of rectal HIV transmission. However, challenges remain namely concerning which properties are more suited for enhancing colorectal distribution and retention of candidate microbicide compounds. In this work, we developed and assessed the *in vitro* and *in vivo* performance of poly(lactic-co-glycolic acid) (PLGA)-based nanoparticles (NPs) as carriers for the model drug efavirenz (EFV). We particularly focused on the effects of non-covalent polyethylene glycol coating of PLGA NPs (PEG-PLGA NPs) conferring mucus diffusive behavior on the pharmacokinetics (PK) of EFV following rectal administration to mice. Drug-loaded PLGA-NPs and PEG-PLGA NPs (200-225 nm) were obtained by nanoprecipitation. Both type of systems were able to retain native antiretroviral activity of EFV, while featuring lower cytotoxicity against different epithelial cell lines and HIV target cells. Also, PLGA-NPs and PEG-PLGA NPs were shown to be readily taken up by colorectal cell lines, and mildly reducing EFV permeation while increasing membrane retention in Caco-2 and Caco-2/HT29-MTX cell monolayer models. When administered to mice in phosphate buffered saline (pH 7.4), EFV-loaded PEG-PLGA NPs consistently provided higher drug levels in colorectal tissues and lavages as compared to free EFV or drug-loaded PLGA-NPs. Mean values for the area-under-the-curve between 15 min and 12 h following administration were particularly higher for PEG-PLGA NPs in distal and middle colorectal tissues, with relative bioavailability values of 3.7 and 29, respectively, as compared to free EFV (2.2 and 6.0 over PLGA NPs, respectively). Systemic exposure to EFV was reduced for all treatments. NPs were further shown safe after once daily rectal administration for 14 days as assessed by histological analysis of colorectal tissues and chemokine/cytokine assay of rectal lavages. Overall, PEG-PLGA NPs demonstrated to be safe carriers for rectal microbicide drug delivery and able to provide enhanced local PK that could be of value in preventing rectal HIV transmission.

4.2. Introduction

The HIV/AIDS pandemic remains a huge burden to our times with a total estimate of over 36 million people living with the infection worldwide by the end of 2016 (1). Sexual transmission of the virus is accountable for the majority of all new cases and URAI represents a considerable fraction of these (2). Although expected to occur less frequently than vaginal intercourse, the higher risk of viral transmission across the colorectal mucosa as compared to the cervicovaginal mucosa (around 10-fold) (3), as well as its practice among both MSM and heterosexual women (4, 5), makes URAI a relevant contributor to the overall number of new infections. Oral PrEP with TDF/FTC for at high risk individuals has been successfully introduced in various countries over the last years and is generally considered as highly effective in preventing sexual HIV transmission (6). Notwithstanding, oral PrEP presents several caveats, raising issues concerning potential ARV resistance, systemic toxicity, inconvenient dosage regimens (once daily, irrespective of sexual activity frequency) potentially leading to poor adherence, suboptimal levels at ano-genital tissues despite high systemic levels, and promotion of risky behavior, among others (7, 8). Cost may also impair large scale population coverage, particularly in resource poor regions (6).

Topical PrEP with rectal microbicides may be an appealing alternative or even auxiliary strategy to oral PrEP (9), in preventing rectal HIV transmission. Rectal microbicides comprise products intended to be self-administered in the rectum around the time of sexual intercourse (10). In particular, microbicides present the potential to provide a new on-demand protection tool that is able to rapidly yield high drug levels at the colorectum with minimal systemic exposure. However, efforts in developing rectal microbicides have been scarce and often limited by the inability of conventional products – typically designed as gels, suppositories or enemas – to allow safe and effective colorectal drug distribution and retention for prolonged time periods (11). Alongside other potentially beneficial features (*e.g.*, controlled drug release, enhanced interaction with HIV-susceptible host cells), nanotechnology-based microbicide drug carriers, may help circumventing drug distribution/retention at the colorectum, thus enhancing local PK. Such an effect has been shown previously for polymeric NPs containing different ARV drugs and administered by the vaginal

route (12, 13). However, it is not clear whether the use of nanocarriers could have an analogous outcome in the case of rectal delivery. Previous *in vivo* work by our group and others supports that the behavior of mucus-penetrating nanosystems administered into the rectum may be particularly beneficial. Dense surface coating of polymeric NPs with PEG was shown not only to contribute to the extensive coverage of the colorectal epithelial surface (14, 15) but also enhance retrograde transport into the proximal end of the colon and provide overall longer particle residence (16).

In the present work, we developed PLGA-based NPs containing the NNRTI EFV and characterized obtained nanosystems, either coated or not with PEG, for physicochemical and *in vitro* biological properties deemed relevant for rectal microbicide development. We further tested *in vivo* safety and the ability of both types of NPs to modify the local PK of EFV as compared to the free drug by using a mouse model.

4.3. Materials and Methods

4.3.1. Materials

End-capped PLGA with 50:50 D,L-lactide:glycolide ratio and 0.2 dL g^{-1} inherent viscosity (Purasorb PDLG 5004) was kindly provided by Corbion (Gorinchem, The Netherlands), EFV (315.68 g mol^{-1}) by BDR Lifesciences (Vadodara, India) and poloxamer P407 (Kolliphor® P 407) by BASF (Ludwigshafen, Germany). Coumarin 6 (C6), polysorbate 80 (Tween® 80), and phosphate buffered saline (PBS) tablets were obtained from Sigma-Aldrich (Schnelldorf, Germany and Lyon, France, respectively). Acetone was purchased from Fisher Scientific (Leicestershine, UK). Ultrapure water used was obtained in-house using a Milli-Q purification system (Merck Millipore, Darmstadt, Germany). All other solvents and chemicals were of analytical grade or equivalent.

4.3.2. Production and physicochemical characterization of nanoparticles

PLGA-based NPs were produced by a nanoprecipitation method as described previously (16). Briefly, 20 mg of PLGA were dissolved in 1 mL of acetone and added to 20 mL of an aqueous solution of poloxamer 407 (0.1%, *w/v*) under magnetic stirring by slowly injecting it using a syringe coupled with a 23G needle. When relevant, one milligram of EFV or 0.08 mg of C6 were co-dissolved with PLGA in acetone in order to obtain EFV-loaded NPs or fluorescent NPs, respectively. In all cases, NPs were collected after 3 h of stirring at room temperature and washed twice with 10 mL of water using a filter tube with a MW cut-off of 100 kDa (Amicon Ultra filter, Ultracel membrane with 100,000 MWCO, Millipore Corporation, Bedford, MA, USA) at 167 ×*g*. NPs were incubated for 12 h with 1% (*w/v*) poloxamer 407 aqueous solution in order to obtain PEG-PLGA NPs. NPs suspensions were centrifuged one more time as described above in order to remove excess poloxamer and to concentrate NPs. Non-modified NPs (PLGA NPs) were treated in the same way by replacing the poloxamer solution by water. NPs were further freeze-dried whenever. NPs dispersed in 10 mM sodium chloride at approximately 0.2 mg mL^{-1} were characterized at 25 ˚C regarding size, size distribution and ZP by using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). The association efficiency (AE) and drug loading (DL) of EFV into NPs were calculated as:

$$AE\ (\%) = \frac{Initial\ EFV - Recovered\ EFV}{Initial\ EFV}\ x\ 100 \qquad \text{(Eq. 4.1)}$$

$$DL\ (\%) = \frac{Initial\ EFV - Recovered\ EFV}{Initial\ EFV\ \&\ PLGA}\ x\ 100 \qquad \text{(Eq. 4.2)}$$

where *Initial EFV* and *Initial EFV & PLGA* are the amounts of drug and drug plus polymer used to produce NPs, respectively. *Recovered EFV* refers to the amount of drug recuperated from filtrates collected during purification of NPs and quantified by

an high-performance liquid chromatography with UV detection (HPLC-UV) method as described in 4.3.2.1.

Surface morphology of NP samples treated with uranyl acetate was assessed by TEM using a JEM-1400 Electron Microscope (JEOL, Tokyo, Japan) at 80 kV. *In vitro* drug release was performed under sink conditions by placing NPs (corresponding to 213 µg of EFV) into 15 mL of 3% (*w/v*) polysorbate 80 in PBS (pH 7.4) at 37 °C and 100 rpm orbital shaking. Release medium samples (0.2 mL) were collected periodically from 15 min to 6 h, centrifuged at 15,000 $\times g$ and the supernatant assayed for EFV by HPLC-UV. Initial volume was replenished with fresh release medium.

4.3.2.1. HPLC-UV method for efavirenz assay

HPLC-UV was used to quantify EFV in different matrices. A reversed-phase method was developed in isocratic mode using a C8 reverse-phase analytical column (Symmetry® LC, 5 µm particle size, 4.6 mm internal diameter, 25 cm length; Waters, Milford, MA, USA). The mobile phase consisted of acetonitrile (ACN) and 10 mM acetate buffer solution pH 4.0 (70:30, *v/v*). The flow rate was maintained at 1 mL min^{-1} and the injection volume was 10 µL. EFV was detected at 247 nm with retention time of 7.2 min. Calibration curves were generated from known standards of EFV in a mixture of ACN and water (1:1). The method was determined to be linear in the range of 1.0 to 50.0 µg mL^{-1} ($r^2 \geq 0.999$ for linear least squares regression). For samples resulting from *in vitro* permeability experiments, injection volumes were increased to 50 µL and linearity established from 0.1 to 10 µg mL^{-1} ($r^2 \geq 0.999$). Also, the mobile phase needed to be adjusted to 43:57 (*v/v*) since polysorbate 80, the surfactant used to assure sink conditions, has a broad spectrum of UV-Vis absorption and affected the EFV peak at original conditions (17). In this case, the retention time of EFV was 13.8 min.

4.3.3. Cell lines and culture conditions

Human colonic epithelial Caco-2 (C2BBe1 clone) cell line was purchased from ATCC (Manassas, VA, USA) and used at passages 54-63. Human colonic epithelial HT29-MTX (passages 27-43) and SW480 (passages 13-15) cell lines were kindly provided by Dr. T. Lesuffleur (INSERM U178, Villejuif, France) and Dr. M. Grade (University of Göttingen, Germany), respectively. TZM-bl cells were acquired from NIH AIDS Reagent Program (Germantown, MD, USA). PBMCs were isolated from buffy coats of healthy adult donors by density gradient centrifugation using Ficoll® Paque Plus (GE Healthcare, Waukesha, WI, USA) followed by stimulation with 5 μg mL^{-1} of phytohemagglutinin (Sigma-Aldrich, St. Louis, MO, USA) in culture medium for 72 h.

Caco-2, HT29-MTX and SW480 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with ultraglutamine (Lonza, Vervieres, Belgium) supplemented with 10% (*v/v*) fetal bovine serum (FBS; Biochrom, Berlin, Germany), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (BioWest, Nuaillé, France), and 1% (*v/v*) non-essential aminoacids, 100× concentrate (Biochrom). TZM-bl were cultured in DMEM supplemented with 10% (*v/v*) FBS, 2 mM L-glutamine, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin, 1 mM sodium pyruvate, and 1 mM non-essential aminoacids, all acquired from Gibco/Invitrogen (Carlsbad, CA, USA). PBMCs were maintained in RPMI-1640 medium (Gibco/Invitrogen) supplemented with 10% (*v/v*) FBS (Gibco/Invitrogen), 2 mM L-glutamine (Gibco/Invitrogen), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Gibco/Invitrogen), 0.3 mg mL^{-1} gentamicin (Gibco/Invitrogen), 5 μg mL^{-1} polybrene (Sigma-Aldrich) and 20 U mL^{-1} recombinant interleukin-2 (NIH AIDS Reagent Program). All cells were maintained at 95% RH, 37 °C and 5% CO_2.

4.3.4. Cytotoxicity of nanoparticles

Cytotoxicity of NPs to epithelial colorectal cell lines Caco-2, HT29-MTX and SW480 was assessed using the triazolyl blue tetrazolium bromide (MTT) metabolic activity assay. Each cell line was individually seeded in 96-well plates at

concentrations of 10^4 cells/well (HT29-MTX and SW480) or 2×10^4 cells/well (Caco-2) for 24 h and further incubated with different concentrations of NPs or free EFV. Media only and 1% (*w/v*) Triton X-100 were used as controls. After 24 h of incubation, cells were washed twice with PBS, pH 7.4, followed by the addition of 200 µL per well of MTT solution (0.5 mg mL^{-1} in medium) and incubation for 4 h. Medium was then removed and 200 µL of dimethyl sulfoxide (DMSO) were added in order to dissolve newly formed formazan crystals. Absorbance was determined at 590 nm with 630 nm background deduction.

In the case of TZM-bl cells and PBMCs, cytotoxicity was determined using the resazurin reduction assay. Cells were individually seeded in 96-well plates at concentrations of 10^4 cells per well (TZM-bl) or 2×10^5 cells per well (PBMCs) and incubated for 24 h. NPs or free EFV were then added at different concentrations for 48 h (TZM-bl) or 14 days with medium being re-freshen at day 3, 7 and 10 (PBMCs). Media only and 5% (*w/v*) sodium dodecyl sulfate (SDS) were used as controls. Afterwards, 10 µL of resazurin solution (alamarBlue™, Invitrogen) were added to each well and cells incubated for an additional 4 h. Fluorescence was determined at 530-560/590 nm.

Experiments were performed in duplicate (TZM-bl cells and PBMCs) or triplicate (Caco-2, HT29-MTX and SW480 cells). Results were calculated as percentage cell viability (cells incubated with medium only considered as 100% viability) and half maximal cytotoxic concentration (CC_{50}) values were calculated from cell viability vs. drug concentration plots using the sigmoidal dose-response (variable slope) model of Prism® (v. 5.01, GraphPad Software, San Diego, CA, USA).

4.3.5. Antiretroviral activity

Antiviral activity of NPs and free EFV was assessed using a Tat-regulated LTR-driven luciferase reporter gene assay in TZM-bl cells as previously described (18). Briefly, TZM-bl cells (10^4 cells/well) were incubated with serial 10-fold dilutions of NPs or free EFV in medium supplemented with 19.7 µg mL^{-1} diethylaminoethyl-dextran for 1 h before infection with 400 $TCID_{50}$ (half-maximal tissue culture infectious

dose) of HIV-$1_{SG3.1}$ or 200 $TCID_{50}$ HIV-1_{NL4-3} isolates (19). NPs or free EFV were present during incubation with the virus. After 48 h of infection, luciferase expression was determined using the Pierce™ Firefly Luc One-Step Glow Assay Kit (Thermo Scientific, Waltham, MA, USA) according to the instructions of the manufacturer. The relative light units (RLUs) were measured using a microplate luminescence reader (Infinite® M200; Tecan, Grödig, Austria). Cells non-exposed or exposed to the virus were used as the 100% and 0% inhibition controls, respectively, in the absence of EFV. Two independent experiments were performed for each compound and each assay was done in triplicate wells. The half-maximal and 90% inhibitory concentrations (IC_{50} and IC_{90}, respectively), as well as the dose-response curve slopes (Hill slope), were estimated by plotting the percent inhibition of infection (*y*-axis) against the log_{10} drug concentration (*x*-axis) using the sigmoidal dose-response (variable slope) equation in Prism® 5.01 software (GraphPad Software, La Jolla, CA, USA). Complementary cell viability assays using the resazurin reduction assay as detailed in 4.3.4. were performed for TZM-bl cells in order to calculate CC_{50}, SI (defined as the CC_{50}/IC_{50} ratio) and relative selectivity index (RSI; defined as the SI of NPs/SI of free EFV ratio) values.

4.3.6. Cell uptake of nanoparticles

Uptake of fluorescent NPs by Caco-2 and HT29-MTX cells was assessed qualitatively and quantitatively by fluorescence microscopy and flow cytometry, respectively. Caco-2 or HT29-MTX cells were seeded in 6-well plates ($0.5 - 1.0 \times 10^6$ cells per well) and on top of a microscope coverslip. After 24 h in culture, fluorescent NPs were dispersed in medium at a concentration of 0.01% (*w/v*) and incubated with cells for 15 min and 2 h. Cells were then washed twice with PBS, stained with CellMask™ Orange (Life Technologies, Eugene, OR, USA), re-washed with PBS and fixed with 2% (*v/v*) paraformaldehyde. Staining with DAPI (Sigma-Aldrich) was further performed. Coverslips containing adherent cells were mounted in slides using Vectashield® antifade mounting media (Vector Laboratories, Burlingame, CA, USA). Qualitative assessment of cellular uptake was performed using an Axiovert 200M inverted fluorescent microscope (Carl Zeiss, Göttingen, Germany). Cross-sectional

imaging of cells along the *z*-axis was performed in order to better assess NPs localization, namely to differentiate between actual intracellular uptake and simply cell membrane adsorption. In the case of quantitative evaluation, cells were incubated with NPs similarly to qualitative studies but without using coverslips. After incubation and twice PBS washing, cells were collected using trypsin-EDTA (Sigma-Aldrich, Lyon, France), transferred to 1.5 mL Eppendorf tubes and centrifuged at 520 $\times g$ (7 min, 4 °C). Cell pellet was washed with PBS, fixed with 2% (*v/v*) paraformaldehyde, re-washed with PBS and finally re-suspended in PBS before storage at 4 °C until further analysis. The quantification of cell-associated fluorescent signal was performed by flow cytometry using a BD FACS Calibur™ system (BD Biosciences, Franklin Lakes, NJ, USA). Data were analyzed using the FlowJo® software v10.0.7 (Tree Star, Ashland, OR, USA).

4.3.7. Drug permeability and retention in colorectal cell monolayers

Permeability and retention of EFV, either free or associated to NPs, in Caco-2 and in Caco-2/HT29-MTX co-culture cell monolayer models was assessed as previously described (20). Briefly, Caco-2 cells alone or in combination with HT29-MTX cells in a proportion of 90:10 were seeded at a density of 10^5 cells cm^{-2} onto 3.0 µm transparent polyethylene terephthalate membrane Transwell® inserts with a cell growth area of 0.90 cm^2 (Corning, Durham, NC, USA). Cells were maintained in culture for 21 days for monolayer formation and maturation, with medium being changed twice weekly. Growth and integrity of mono- and co-culture monolayers was monitored by measuring the transepithelial electrical resistance (TEER) using an EVOM^2 voltohmmeter equipped with a STX2 electrode (World Precision Instruments, Sarasota, FL, USA). TEER values were calculated by subtracting the resistance of a blank (cell-free culture insert with medium) to experimental values, and correcting for the surface area. On the day of experiments, the medium was removed from the apical and basolateral compartments and the cells were washed twice with Hanks' Balanced Salt Solution (HBSS; Gibco, Waltham, MA, USA). Fresh HBSS was added to both compartments and the system was allowed to equilibrate for 30 min at 37 °C. HBSS from the receptor compartment contained 0.2% (*w/v*) poloxamer 407 in order

to maintain sink conditions. Then, HBSS from the apical compartment was replaced by 0.5 mL of EFV-loaded NPs or free EFV dispersed in HBSS at a final drug concentration of 0.016% (*w/v*). Transwell® inserts were kept under orbital shaking (100 rpm) at 37 °C throughout the experiment. TEER was also monitored before experiments and at each time point to assure the viability and cell monolayer integrity during the entire assay. Samples of 200 µL were collected at pre-defined time points from basolateral compartments, assayed for EFV by HPLC-UV as described above, and replaced with the same volume of fresh HBSS. After the last time point, media were removed from both compartments of the Transwell® system and cells were washed twice with HBSS. The insert was isolated from the Transwell® system and EFV extracted from the cell monolayer by immersion in 0.5 mL of DMSO overnight under shaking (150 rpm). The amount of drug recovered from the basolateral (receptor) media from 15 min to 4 h was used to calculate apparent permeability coefficient (P_{app}) values in cm s^{-1} as follows:

$$P_{\mathrm{app}} = \frac{Q}{A \times C_0 \times t}$$

(Eq. 4.3)

where *Q* is the total amount of drug that permeated the cell monolayer (µg), *A* is the diffusion area (cm^2), C_0 is the initial concentration of drug (µg cm^3), and *t* is the time of experiment (s). The coefficient *Q*/*t* represents the steady-state flux of drug across the cell monolayer. The permeability enhancement ratio (*PER*) was calculated by dividing mean values of P_{app} of EFV when associated to NPs by P_{app} of the free drug. The amount of EFV associated to cell monolayers was also determined at the end of the experiment.

4.3.8. Pharmacokinetics and safety studies

All animal experiments were approved by the Ethics Committee at Instituto Universitário de Ciências da Saúde (process no. 01/ORBEACESPU/2014) and

conducted under European Directive 2010/63/EU guidance. Male CD-1 IGS mice (Harlan, Barcelona, Spain) were used between 8-12 weeks old.

Local and systemic PK of EFV were assessed over 12 h after rectal administration of EFV-loaded NPs or free drug at a total dose of 40 μg of EFV. Animals were fasted for 24 h before experiments, with free access to water, and the rectum was gently flushed with 200 μL of deionized water at 30 min prior to rectal administration in order to reduce and soften the consistency of fecal content. Timing of this last procedure also allows for the mucus layer to be replenished before administration of NPs or EFV (14). NPs or the free drug were dispersed in isotonic PBS (pH 7.4) and 20 μL (corresponding to 40 μg of EFV) administered intrarectally using a 200 μL pipette tip. Mice were sacrificed at pre-defined time points from 15 min to 12 h following administration by overdose of inhalational isoflurane followed by intracardiac exsanguination. Blood was collected into Vacuette® tubes containing K_3EDTA (Greiner Bio-One GmbH, Kremsmünter, Austria), centrifuged (496 ×*g*, 10 min, 4 °C) and the plasma recovered. Proximal, middle and distal/rectum segments of the colon were collected, weighted and individually washed with 350 μL of PBS. Plasma, as well colon tissue segments and washing fluids, were stored at -80 °C until further processing. All biological samples were processed and analyzed by a HPLC method coupled to tandem mass spectrometry detection (HPLC-MS/MS) method (see section 4.3.8.1).

Safety of NPs was assessed upon once daily rectal administration for 14 days to mice. NPs or the free drug were administered intrarectally as indicated for PK studies. Mice treated similarly with PBS only, 2% (*w/v*) N-9 (21) or without any treatment were used as controls. Animals were monitored daily for discomfort or behavioral changes, body weight and macroscopic alterations at the anal region. The rectum was flushed with 5×50 μL of PBS at 30 min prior to each administration, and lavage samples were centrifuged (13,414 ×*g*, 10 min, 4 °C) and supernatants stored at -80 ˚C until further use. The same procedure was also performed following mice sacrifice (inhalational isoflurane overdose), 24 h after the last administration. The colon was also collected following euthanasia, roughly divided in three equal sized segments (proximal, middle and distal/rectum) as previously described, processed for hematoxylin and eosin (H&E) staining, and analyzed for histological features.

Rectal lavages were analyzed for interleukin (IL)-1β, IL-6, interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) levels by an ELISA-based chemiluminescent multiplex assay (Quansys Q-Plex™ Cytokine Array, tebu-bio, Le Perray en Yveline, France). All treatments were performed in three animals.

4.3.8.1. Quantification of efavirenz from biological samples by HPLC-MS/MS

The quantification of EFV in samples resulting from mouse studies was performed by using a previously described and validated HPLC-MS/MS (22), as briefly detailed in the following. The mobile phase contained two components, namely 0.1% *(v/v)* formic acid in water and 0.1% (*v/v*) formic acid in ACN that were filtered through a 0.22 µm Millipore GVWP filter and a 0.45 µm Millipore HVHP filter, respectively, and degassed in an ultrasonic bath for 15 min before use. EFV purchased from Sigma-Aldrich and deuterated efavirenz-d_5 (EFV-d_5) obtained from Toronto Research Chemicals Inc. (Toronto, ON, Canada), through LGC standards (Barcelona, Spain) were used as standard and internal standard (IS), respectively. HPLC-MS/MS analysis was performed in a Nexera X2 UHPLC system coupled to a triple quadrupole LCMS-8040 mass spectrometer equipped with an electrospray ionization source (ESI) (Shimadzu Corporation, Kyoto, Japan). Chromatographic separation was achieved using a reversed phase Mediterranea sea C18 column (3 µm, 100 x 2.1 mm; Teknokroma, Barcelona, Spain) at 45 °C and elution was performed in gradient mode, at total flow rate of 0.35 mL min^{-1}, according to the program established previously (22). During each chromatographic run, the column eluate was directed to MS interface between 3.60 and 4.55 min. The mass spectrometer was operated in negative ionization mode (ESI-) and data were acquired in selected reaction monitoring (SRM) mode (EFV, *m/z* 314.00 > 68.95, *m/z* 314.00 > 244.05; EFV-d_5, *m/z* 319.00 > 68.90, *m/z* 319.00 > 247.95). Calibration curves were established with EFV standard solutions prepared at final concentrations of 4, 10, 20, 50, 100, 200, 350 and 500 ng mL^{-1} in ACN-water (50:50, *v/v*). Prior to analysis, tissue extracts in ACN were evaporated to dryness under a gentle stream of nitrogen and re-suspended in 500 µL of ACN-water (50:50, *v/v*). Lavage and blood

plasma extracts were diluted 1:5 using the same solvent. EFV-d_5 was added to each standard solution and sample extract in order to reach the final concentration of 400 ng mL^{-1}. The injection volume was 5 µL.

Matrix effect was evaluated for EFV and corresponding IS by comparing the peak area obtained in spiked blank matrix extracts (200 ng mL^{-1} of EFV and 400 ng mL^{-1} of EFV-d_5) with the peak area obtained for the same concentration of analytes in ACN-water (50:50, *v/v*) (**Table 4.1**). The extraction efficiency of target analyte and IS was evaluated for the different biological matrices (**Table 4.2**). The recovery from colorectal tissues was assessed by spiking the tissues with 1 µg of EFV and 2 µg of EFV-d_5 per g of matrix, before extraction. In the case of lavage and plasma samples, 2 µg of EFV and 4 µg of EFV-d_5 were added per mL of fluid before sample processing. The percentage of recovery was obtained by comparing the peak area of spiked samples with the peak area of standard solutions prepared at the same concentration.

Table 4.1. Matrix effect evaluation for the analysis of EFV in tissue, lavage and plasma extracts.

Matrix	Matrix factor (EFV)[a]		Matrix factor (IS)[a]		IS normalized matrix factor[a,b]	
	Mean (%)	CV (%)	Mean (%)	CV (%)	Mean (%)	CV (%)
Distal colon tissue	93.6	7.0	97.5	6.1	96.0	0.9
Middle colon tissue	93.8	0.8	100.3	1.9	93.5	1.1
Distal colon lavage	94.6	4.5	98.2	7.4	96.4	2.9
Middle colon lavage	97.2	0.5	97.0	1.0	100.3	0.5
Blood plasma	91.8	0.1	94.1	1.7	97.6	1.6

[a] The matrix effect was evaluated for two different blanks of each biological matrix (n=2); [b] IS normalized matrix factor was calculated by division of the matrix factor of the analyte by the matrix factor of the IS.

Limits of detection (LOD) and quantification (LOQ) of EFV for each matrix were determined as the concentration values that originated a signal-to-noise ratio in analytical chromatograms of 3:1 and 10:1, respectively (**Table 4.3**). Different PK parameters were determined namely maximum concentration (C_{max}) and time at

which it occurred (t_{max}), area under the concentration-time curve between 15 min and 12 h ($AUC_{0.25\text{-}12h}$) and relative bioavailability (F_{rel}) as compared to the free drug, as previously described (13). Groups of five animals were used for all experimental conditions (treatments and time points).

Table 4.2. Extraction efficiency of EFV and corresponding IS from biological matrices.

Matrix	Target concentration on final extract (ng mL^{-1})	Recovery (EFV)[a]		Recovery (IS)[a]	
		Mean (%)	CV (%)	Mean (%)	CV (%)
Distal colon tissue	200	95.2	3.2	99.6	1.6
Middle colon tissue	200	91.2	7.0	94.6	4.3
Distal colon lavage	200	92.6	9.6	95.9	5.6
Middle colon lavage	200	92.9	5.1	99.8	1.1
Blood plasma	200	98.0	1.8	90.1	2.0

[a] The extraction efficiency of EFV and IS was assessed for three different tissue samples (n=3) and two different lavage and plasma samples (n=2).

Table 4.3. Limits of detection (LOD) and quantification (LOQ) of EFV determined for tissues, fluids and blood plasma.

	LOD (µg.L^{-1})	LOQ (µg.L^{-1})
DC fluid	6	12
MC fluid	5	11
DC tissue	4	8
MC tissue	3	7
Blood plasma	3	8

4.3.9. Statistical analysis

Analysis was performed in Prism® 5.01 software using Student's *t*-test for two group comparisons or one-way ANOVA with Bonferroni's Multiple Comparison Test. Experiments were performed in triplicate, except if noted otherwise, and values of $p<0.05$ were accepted as denoting significance. Data is presented as mean ± SD values, except for *in vivo* results in which error is described as the standard error of the mean (SEM).

4.4. Results and Discussion

4.4.1. Production and physicochemical properties of nanoparticles

The major motivations underlying this work were to explore the potential benefit of nanotechnology for the development of microbicide drug carriers and to contribute for the ongoing debate regarding the use of either mucoadhesive or mucus-inert nanosystems for mucosal drug delivery (23). In order to pursuit these goals, we proceeded with the production of EFV-loaded PLGA NPs followed by surface modification with poloxamer 407. EFV was chosen as a model microbicide drug not only because of its intrinsic potent ARV activity and acceptable safety profile, but also due to its intrinsic physicochemical properties, namely low aqueous solubility, which are challenging in terms of more conventional formulation in aqueous media. Furthermore, EFV shares these attributes and limitations with other drugs that are in more advanced stages of microbicide development, particularly dapivirine (24), thus making it a good surrogate. The choice of nanocarrier type and its manufacturing technique took into consideration factors such as regulatory status, availability and affordability of used materials, as well as potential for manufacturing scalability, in order to yield systems that could realistically move forward as putative anti-HIV microbicide candidates.

EFV-loaded PLGA NPs were successfully produced by nanoprecipitation and further modified using poloxamer 407. Incubation of polymeric NPs with this type of

triblock copolymers comprising PEG and PPG chains (PEG-PPG-PEG) allows obtaining nanosystems featuring a non-covalent PEG corona (25). In particular, poloxamer 407 used at 1% (*w/v*) is able to confer densely PEG-coated NPs that hinder interactions with mucus (26) and, more importantly, enhance the distribution and retention of these nanosystems following rectal administration in mice (16). Obtained EFV-loaded PLGA NPs and EFV-loaded PEG-PLGA NPs, as well their blank and C6-loaded (fluorescent) counterparts, presented average hydrodynamic diameters around 200-225 nm and narrow size distribution (**Table 4.4**). Main differences concerned ZP, varying from slightly negative for PLGA NPs to near neutral for PEG-PLGA NPs, respectively. The near neutral charge for PEG-PLGA NPs is indicative of the dense coating of NPs surface with poloxamer 407 as previously demonstrated (16).

Table 4.4. Physicochemical properties of NPs. Hydrodynamic diameter (HD), polydispersity index (PdI), zeta potential (ZP), association efficiency (AE) and drug loading (DL) of EFV-loaded PLGA NPs. Results are presented as mean ± SD (*n*=3).

Nanosystem	Payload	HD (nm)	PdI	ZP (mV)	AE (%)	DL (%)
PLGA NPs	EFV	217 ± 4	0.072 ± 0.002	-6.6 ± 0.6	85.9 ± 2.6	4.3 ± 0.1
	None	200 ± 2	0.087 ± 0.006	-6.6 ± 0.9	–	–
	C6	221 ± 10	0.087 ± 0.029	-4.7 ± 0.1	ND	ND
PEG-PLGA NPs	EFV	223 ± 3	0.101 ± 0.009	-2.0 ± 0.1	83.2 ± 3.6	4.2 ± 0.2
	None	204 ± 5	0.070 ± 0.009	-2.0 ± 0.3	–	–
	C6	216 ± 2	0.075 ± 0.004	-1.9 ± 0.1	ND	ND

ND: not determined.

Association of EFV was high for both types of NPs (>80%), resulting in total DL values of around 4%. TEM imaging revealed the spherical shape of NPs and confirmed size and size distribution values obtained by DLS (**Figure 4.1, A-D**). Fast release of EFV from NPs was observed under sink conditions (nearly 60% of total payload within the first hour), followed by slow release up to at least 6 h (**Figure 4.1, E**). Although often seen as detrimental, such burst release following administration may be biologically relevant given the typical use of rectal microbicides as on-

demand products (*i.e.*, around the time of sexual intercourse) and the need for at least partial immediate availability of the drug for exerting its antiviral activity.

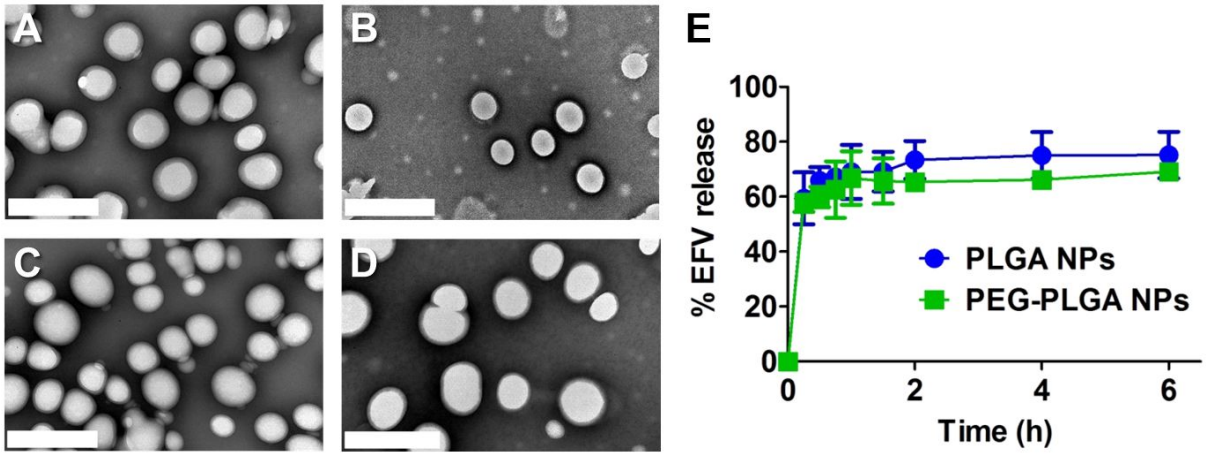

Figure 4.1. Morphology and *in vitro* drug release profile of NPs. Representative TEM images of **(A)** EFV-loaded PLGA NPs, **(B)** EFV-loaded PEG-PLGA NPs, **(C)** blank (no drug) PLGA NPs, and **(D)** blank PEG-PLGA NPs (scale bars = 500 nm). **(E)** Release profile of EFV from PLGA NPs and PEG-PLGA NPs over 6 h in PBS (pH 7.4) added with 3% polysorbate 80 (*w/v*). Results are presented as mean ± SD (*n*=3).

4.4.2. Cytotoxicity and antiretroviral activity of nanoparticles

Assuring the potential safety of delivery systems is essential even at early stages of the microbicide development pipeline and this can be performed by using relevant cell-based models (10). Since EFV induces cytotoxic effects mainly due to oxidative stress at the mitochondria (27), MTT and resazurin tests were selected for the present study. The *in vitro* metabolic activity of different colorectal epithelial cell lines (Caco-2, HT29-MTX and SW480) and PBMCs, used as proxy for HIV target cells, was assessed following exposure to free EFV or drug-loaded NPs. The CC_{50} values calculated from viability vs. drug concentration plots are presented in **Table 4.5**. The presence of both types of NPs appeared to have no toxic effects on tested cells over the range of concentrations evaluated and up to 100 μM (expressed as EFV concentration). In contrast, free EFV was able to decrease cell viability at lower

concentrations, featuring CC_{50} values typically in the range of 10-20 μM. Such apparent reduction of the cytotoxicity of EFV upon association to nanocarriers has been reported (28, 29) and may be related to the lower amount of free compound for triggering toxic effects at the cytoplasm level.

Table 4.5. Cytotoxicity potential of EFV-loaded NPs. CC_{50} values in μM and expressed as EFV content for the free drug and drug-loaded NPs when tested using different colorectal epithelial cell lines and PBMCs. Results are presented as mean ± SD (*n*=2-3).

	Caco-2	HT29-MTX	SW480	PBMCs
EFV	14.4 ± 2.3	14.2 ± 2.0	16.3 ± 5.1	27.0 ± 5.9
PLGA NPs	>100	>100	>100	>100
PEG-PLGA NPs	>100	>100	>100	>100

We further conducted *in vitro* assays in order to assess the impact of using EFV-loaded NPs on TZM-bl cell infection by two HIV-1 strains. Results for IC_{50} and IC_{90} are presented in **Table 4.6** and show that both types of EFV-loaded NPs displayed potent antiviral activity which was only slightly lower than the free drug. Importantly, drug-loaded NPs displayed markedly lower toxicity to TZM-bl cells as compared with the free drug (CC_{50} = 22 ± 1 μM), thus rendering overall higher SI values and being indicative of better safety profiles. Interestingly, EFV-loaded PEG-PLGA NPs also presented mildly higher CC_{50} values in TZM-bl cells than EFV-loaded PLGA-NPs (>1,000 μM vs 576 ± 47 μM).

Table 4.6. Activity of EFV-loaded NPs against HIV-1. Values of half maximal inhibition concentration (IC_{50}) and 90% inhibition concentration (IC_{90}) for free EFV and drug-loaded NPs against infection of TZM-bl cells by HIV-$1_{SG3.1}$ or HIV-1_{NL4-3}. Results are expressed as EFV concentration and presented as mean ± SD (*n*=2). Selectivity index (SI) and relative selectivity index (RSI) values are dimensionless and were calculated using mean IC_{50} and CC_{50} values.

	HIV-$1_{SG3.1}$				HIV-1_{NL4-3}			
	IC50 (nM)	IC90 (nM)	SI	RSI	IC50 (nM)	IC90 (nM)	SI	RSI
EFV	0.12 ± 0.07	22 ± 10	0.2×10^6	–	0.001 ± 0.001	45 ± 15	16×10^6	–
PLGA NPs	0.98 ± 0.30	32 ± 10	0.6×10^6	3.2	0.018 ± 0.022	68 ± 15	32×10^6	1.9
PEG-PLGA NPs	0.73 ± 0.22	37 ± 2	$>1.4\times10^6$	>7.4	0.022 ± 0.024	74 ± 13	$>45\times10^6$	>2.7

4.4.3. Nanoparticle uptake and drug permeability/retention in epithelial colorectal cell-based models

We proceeded our investigation by further studying the interactions between NPs and epithelial cells. Although not susceptible to viral infection, such cells comprise the bulk of the colorectal lining, thus potentially participating indirectly in HIV transmission (*e.g.*, occurrence of breaches that provide direct access of the virus to the underlying lamina propria) and having an important role in determining local and systemic PK. Uptake studies by epithelial cells were conducted using fluorescent NPs. The incorporation of C6 did not affect substantially the colloidal properties of matching NPs (**Table 4.4**). Also, leaching of the fluorescent dye was determined to be minimal as tested *in vitro* under sink conditions (<4% after 24 h in PBS pH 7.4 at 37 °C), which essentially assures its co-localization with NPs. Uptake occurred rapidly for both PLGA NPs and PEG-PLGA NPs, either by enterocyte-like Caco-2 or mucus-producing HT29-MTX cells, as assessed by fluorescent microscopy (**Figure 4.2, A-B & D-E**). Images from *z*-stacking confirmed cell internalization of NPs that seemed to be mainly localized at the cytoplasm. Data from flow cytometry analysis indicated that the uptake of both type of particles increased over time but was generally higher in the case of PEG-PLGA NPs (around 2- to 4-fold increase over PLGA NPs). Reasons for such distinct differences are not clear. For instance, the production of mucus by HT29-MTX cells in the reduced timeframe of the experiment does not seem to be sufficient to yield a relevant barrier to PLGA NPs diffusion (30). One possible explanation for enhanced uptake of PEG-PLGA NPs may reside in the ability of poloxamers to interfere with the microviscosity/fluidity of cell membranes by a mechanism that is not completely understood (31).

The impact of NPs on permeability and retention of EFV was also assessed by using two cell monolayer models developed by our group (20). Both models are based on colorectal Caco-2 cells, reflecting the basic structure of the colorectal epithelium. Furthermore, the incorporation of HT29-MTX cells allows sustaining the production of mucus (32). Drug permeability profiles across both were used to calculate P_{app} and *PER* values, as presented in **Table 4.7**.

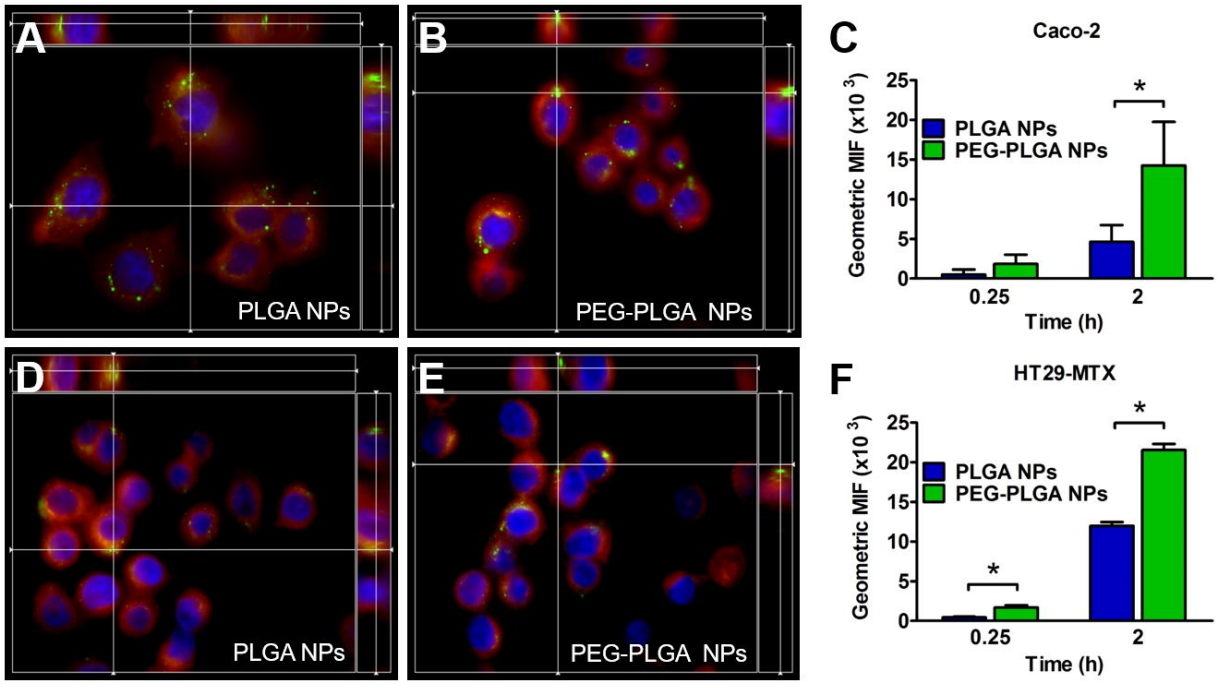

Figure 4.2. Qualitative (fluorescence microscopy) and quantitative (flow cytometry) assessment of NP uptake by **(A-C)** Caco-2 and **(D-F)** HT29-MTX colorectal cell lines. Representative microscopy images were acquired at magnification ×100 after 15 min of incubation. Range in *z*-axis is 7 μm. Green, blue and red signals are from C6 (associated with NPs), DAPI (DNA) and CellMask™ Orange (cell membrane), respectively. Data for flow cytometry are presented as mean ± SD (*n*=3). (*) denotes a significant difference ($p<0.05$).

Results for the free drug are in general agreement with those previously published, with differences likely related to variations in experimental settings (**Figure 4.3, A-B**) (33, 34). The use of EFV-loaded NPs led to a mild decrease in permeability as compared to the free drug, which could potentially contribute to decreased absorption and systemic bioavailability following *in vivo* administration. These results may reflect the delayed drug availability due to the need for EFV to be release from NPs. Comparison between data for the two models further showed no difference between P_{app} values for free EFV or each NP formulation.

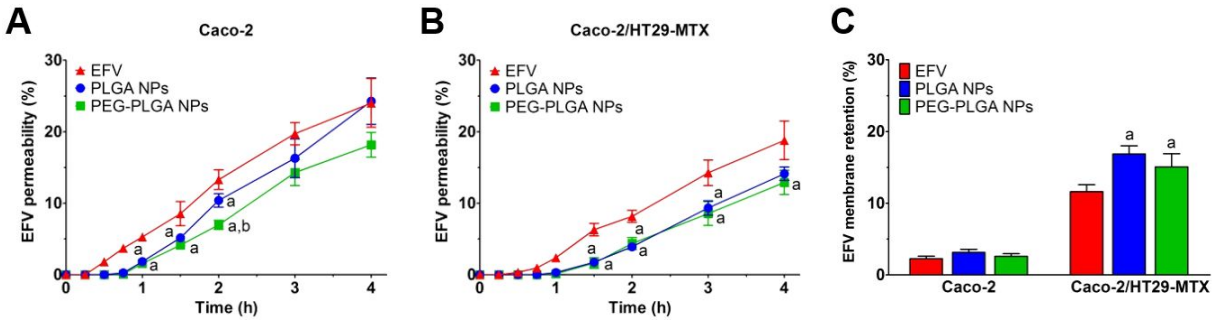

Figure 4.3. Membrane permeability and retention of EFV in colorectal cell monolayer models. Drug permeability profile across **(A)** Caco-2 cell monolayers and **(B)** Caco-2/HT29-MTX cell co-culture monolayers. **(C)** Retention of EFV in Caco-2 and Caco-2/HT29-MTX cell monolayers after 4 h of incubation. Data are expressed as mean ± SD percentage values of initial amount of EFV in the donor (apical) compartment (*n*=3). ([a]) and ([b]) denote a significant difference ($p<0.05$) when compared with the free drug or PLGA NPs, respectively.

Such observations, however, do not necessarily mean that the presence of mucus (although in scarce amounts (32)) does not play a role in drug/NP transport since other factors such as drug release/dissolution dynamics, as well as cell monolayer tightness, NP uptake and drug accumulation may play important roles in the permeability phenomena (35). Regarding the accumulation of EFV at cell monolayers, only mild differences were observed in the co-culture model, with slightly higher retention being observed in the case of NPs (**Figure 4.3, C**). This could reflect the intimate association between NPs and the portion of mucus that was not completely removed during washing. Overall and in the absence of complex luminal transport phenomena occurring only *in vivo* (14-16), data suggest that both types of NPs have little effect on the permeability and retention of EFV.

Table 4.7. Drug permeability across colorectal epithelium models as mediated by NPs. Apparent permeability coefficient (P_{app}) and permeability enhancement ratio (*PER*) of EFV across Caco-2 and Caco-2/HT29-MTX cell monolayers for the free drug and EFV-loaded NPs. Results are presented as mean ± SD (n=3). ([a]) denotes a significant difference (p<0.05) when compared with the free EFV.

	Caco-2		Caco-2/HT29-MTX	
	P_{app} (cm $s^{-1}\times10^{-6}$)	*PER*	P_{app} (cm $s^{-1}\times10^{-6}$)	*PER*
EFV	9.3 ± 1.3	–	7.3 ± 1.0	–
PLGA NPs	4.8 ± 0.6[a]	0.52	5.4 ± 0.4	0.74
PEG-PLGA NPs	4.1 ± 0.4[a]	0.44	5.0 ± 0.7[a]	0.68

4.4.4. Pharmacokinetics of efavirenz-loaded nanoparticles

The potential of NPs for rectal delivery of EFV was further assessed *in vivo* using a murine model. Drug levels in colonic lavages and tissues, as well as in blood plasma, were determined between 15 min and 12 h following administration. The colon was divided into three equally sized segments (proximal, middle and distal), as detailed in **Figure 4.4.**, in order to better understand the lengthwise distribution of EFV. Systemic exposure to EFV was generally low and limited in time following administration (**Figure 4.4, B**). Still, the use of NPs resulted in a mild increase in absorption, as inferred from $AUC_{0.25\text{-}12h}$ values (**Table 4.8**), which was significant in the case of PEG-PLGA NPs (p=0.0014). Previous PK studies in mice are not available for EFV but overall plasmatic concentrations were negligible when compared to those obtained for comparable oral doses in other species, namely rats and Rhesus monkeys (36). The relatively low dose of EFV administered intrarectally (roughly 1% of the amount used for therapeutic purposes if considering the standard oral dose of 600 mg for humans and an interspecies allometric scaling factor of 12.3 (37)), as well as its preferential absorption at the intestinal level (38), justify the reduced systemic exposure to the drug.

Local concentrations are an important predictor of the efficacy of microbicide drugs (39, 40). Findings from our previous study concerning the biodistribution of NPs following intrarectal administration were considered as the ground basis for the

conducted PK work (16). In particular, we showed that ≈200 nm PEG-PLGA NPs were able to reach nearly 40% of the total extent of the colon between 15 min and 2 h after rectal delivery (16). EFV levels in colonic fluids were estimated by lavage in the present work (**Figure 4.4, C-D**). No significant differences were observed for animals treated with either free drug or drug-loaded NPs, except for PEG-modified nanocarriers at 15 min in the distal colon. Results for C_{max} and $AUC_{0.25\text{-}12h}$ denote a trend towards higher levels of EFV in fluids when PEG-PLGA NPs were used (**Table 4.8**), particularly at the middle colon. Variability among individual animals was high and in line with the intense stool trafficking in mice, namely at the distal colon (41), that is unavoidable even when considering fasting and cleaning measures undertaken during animal preparation. Continuous colonic movement and fecal output may also justify the rapid drug clearance observed, within as much as 2 h following administration.

Differences in EFV levels between treatment groups were particularly apparent for tissues (**Figure 4.4, E-F**). Again, variation between intra-group individuals limited significance at individual time points, which was only observed for PEG-PLGA NPs in the distal colon at 15 min. However, analysis of $AUC_{0.25\text{-}12h}$ data (**Table 4.8**) clearly demonstrates that the concentrations of EFV for drug-loaded PEG-PLGA NPs were significantly higher than those for the free drug at both the distal (p=0.0053) and middle (p=0.0179) segments of the colon. Strikingly, a nearly 30-fold increase in drug levels was observed in the case of PEG-PLGA NPs in the middle colon as compared to free EFV. Significantly higher drug concentrations were also noted for PEG-modified NPs as compared to plain PLGA NPs at this colon segment (approximately 6-fold increase; p=0.0405). Such results are in agreement with our previous observations demonstrating that similar PEG-PLGA NPs can enhance retrograde distribution and retention in the colon after intrarectal administration (16). Lastly, we were unable to quantify or detect EFV in the proximal colon at any given time point or treatment group. Again, these observations appear to back up the inability of NPs to distribute past the lower segments of the colon, as well as the low systemic absorption and distribution of EFV after rectal administration that could provide indirect access to the proximal colon.

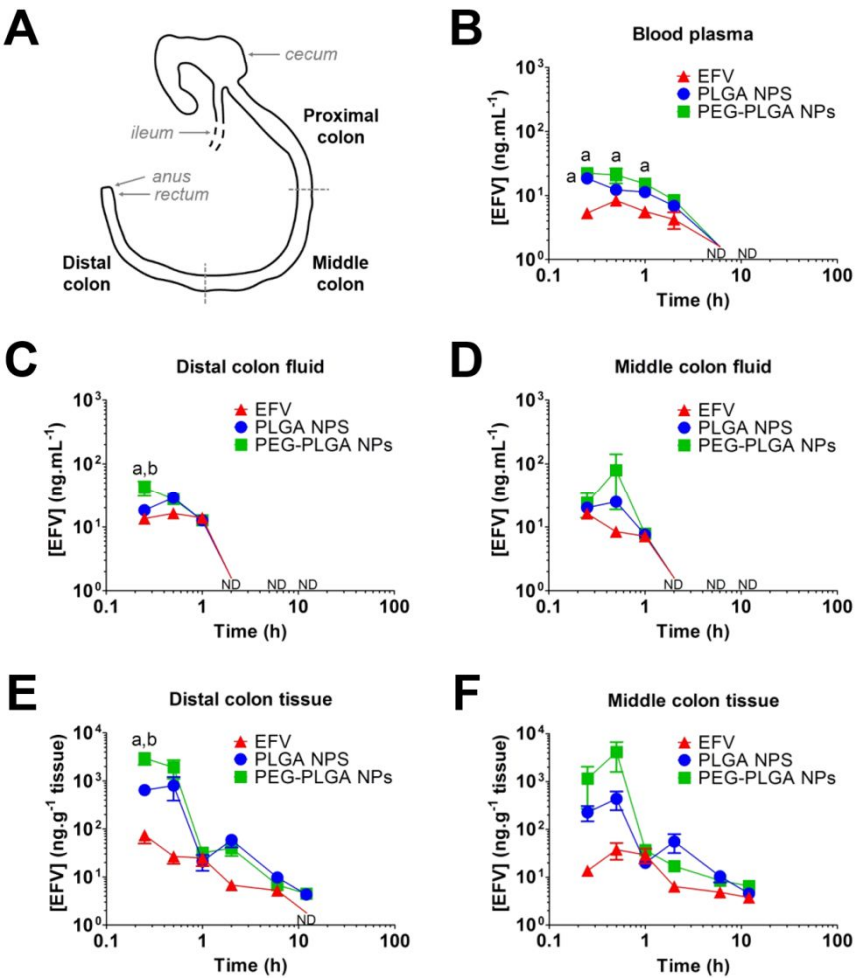

Figure 4.4. Local and systemic PK profiles of EFV following rectal administration of either free drug or EFV-loaded NPs. **(A)** Schematic representation of the murine large intestine and delimitation of the different segments considered for local PK assessment. PK profiles from 15 min to 12 h are presented for **(B)** blood plasma, **(C, D)** distal and middle colon fluids (expressed as lavage levels), and **(E, F)** distal and middle colon tissues. Results are presented as mean ± SEM levels of EFV per volume of fluid (plasma or lavage), or per tissue weight (n=5). (^{a}) and (^{b}) denote significant differences (p<0.05) when compared with the free drug or PLGA NPs, respectively. ND: not detected.

Table 4.8. Local and systemic pharmacokinetics of EFV-loaded NPs. Data are presented for fluids and tissues of distal (DC) and middle (MC) colon segments, as well as blood plasma, upon rectal administration of free EFV or EFV-loaded NPs. Results are expressed as mean ± SEM (n=5). ([a]) and ([b]) denote a significant difference (p<0.05) when compared with free EFV or PLGA NPs, respectively.

	C_{max} (ng mL^{-1} *or* ng g^{-1})			t_{max} (h)			$AUC_{0.25\text{-}12h}$ (ng h mL^{-1} *or* ng h g^{-1})			F_{rel}		
	EFV	PLGA NPs	PEG-PLGA NPs	EFV	PLGA NPs	PEG-PLGA NPs	EFV	PLGA NPs	PEG-PLGA NPs	EFV	PLGA NPs	PEG-PLGA NPs
DC fluid	16.3 ± 2.2	28.7 ± 13.8	41.9 ± 11.1	0.5	0.5	0.25	18.2 ± 0.7	22.6 ± 4.4	25.0 ± 2.9	–	1.2	1.4
MC fluid	16.2 ± 6.9	24.9 ± 7.2	79.8 ± 54.6	0.25	0.5	0.5	10.6 ± 0.8	17.5 ± 2.8	38.8 ± 17.1	–	1.7	3.7
DC tissue	74.2 ± 24.7	798 ± 408	2,969 ± 733	0.25	0.5	0.25	93.0 ± 7.9	605 ± 150	1,258 ± 324[a]	–	6.5	14
MC tissue	37.6 ± 14.4	437 ± 165	4,121 ± 2,265	0.5	0.5	0.5	86.9 ± 13.0	412 ± 62	2,558 ± 906[a,b]	–	4.8	29
Blood plasma	8.4 ± 1.0	18.5 ± 1.6	22.4 ± 1.3	0.5	0.25	0.25	18.6 ± 3.5	32.6 ± 5.1	43.4 ± 1.7[a]	–	1.8	2.3

Overall, our PK results seem to point out for a clear advantage of using drug-loaded polymeric NPs in the context of rectal microbicide development, namely PEG-PLGA NPs. The ability of nanocarriers to better distribute and retain in the lower colon may be particularly relevant in enhancing local drug levels. Still, there are several limitations to the present study that should be considered when analyzing our data. First, difficulties in translating to the human scenario are considerable due to the striking interspecies differences. Mice present shorter GIT transit time and intense peristalsis and stool trafficking (41, 42), which predictably contribute to a shorter timeframe for drug/nanocarrier residence at the colorectum. The use of other animal models that are phylogenetically closer to humans could reduce these limitations. However, our results should be readily translatable into HIV-susceptible humanized mice, which are valuable models in early pre-clinical development of microbicides including in the determination of dose-response relationships (43, 44). Another challenge in our study is the clear distinction between the fractions of EFV that are embedded or simply adsorbed at the surface of tissue. Washing procedures of the different segments of the colon cannot assure the full removal of natural fluids and, thus, tissue levels also account for drug that may be intimately associated with deeper layers of mucus and epithelial surface. This potential bias, however, seem to be of little concern as parallel issues are also found in human clinical trials when collecting biopsies for determining PK/PD correlations of rectal microbicides (45). Lastly, the adopted PK study protocol is unable to distinguish between the fractions of associated and free drug that contribute to the total EFV levels in biological samples from mice treated with drug-loaded NPs. This information could be of relevance in understanding the actual amount of EFV available to inhibit viral infection, namely at the cytoplasm of HIV-susceptible cells. Our *in vitro* drug release studies results seem to point out to the fast release of EFV under sink conditions, which could circumvent any concerns regarding limited availability of free drug *in vivo*.

4.4.5. *In vivo* safety of efavirenz-loaded nanoparticles

Asserting the safety of microbicide candidates is crucial early on during pre-clinical testing. The local effects of both types of EFV-loaded NPs were evaluated after 14 days of once daily rectal administration at the same drug dose used in PK studies. No changes in behavior, body weight, macroscopic appearance of the perianal area, or fecal content were noted throughout the duration of the study in mice treated with NPs, free EFV, PBS or N-9 as compared to non-treated animals. No macroscopic changes at the abdominal cavity were noted during necropsy performed at 24 h after the last administration. Microscopic evaluation of the distal colon of mice treated with free drug or EFV-loaded NPs showed no alterations in tissue architecture as compare to either PBS-treated or non-treated animals (**Figure 4.5**).

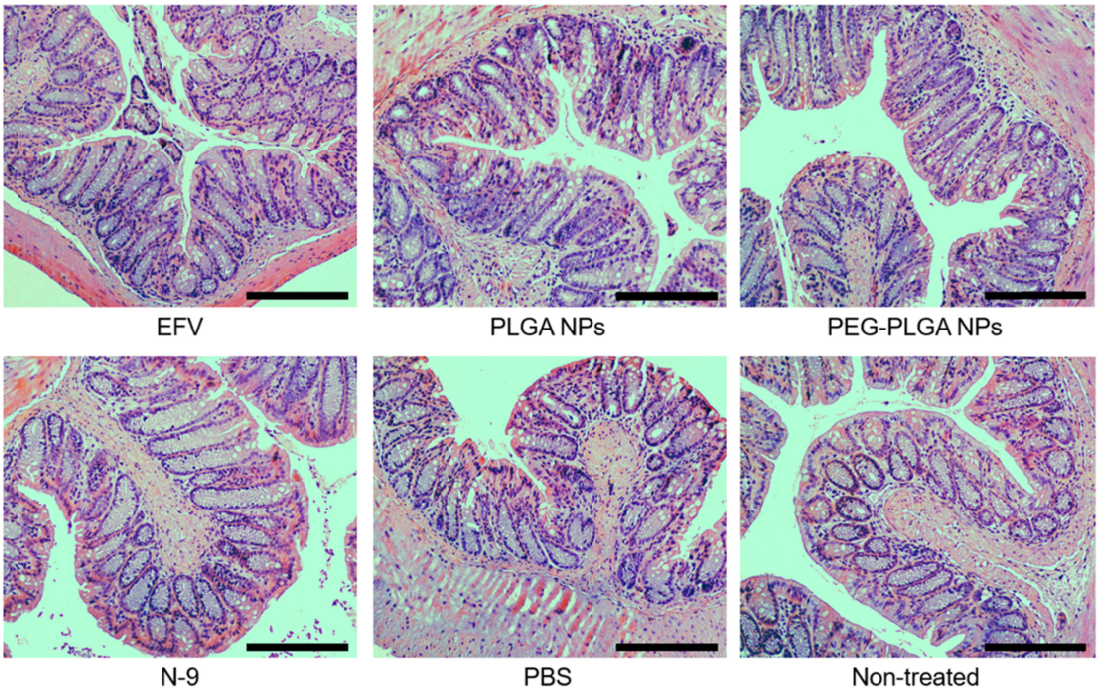

Figure 4.5. *In vivo* safety evaluation of EFV-loaded NPs after once daily rectal administration to mice for 14 days. Representative H&E microphotographs of distal colon sections of mice treated with either free drug, EFV-loaded PLGA NPs or EFV-loaded PEG-PLGA NPs. Control groups included animals administered with N-9 or PBS, as well as mice undergoing no treatment (scale bar=200 μm).

In the case of mice treated with N-9 – one of the first microbicide candidates meanwhile discarded due to its toxic effects to the rectal mucosa that presumably lead to enhanced HIV transmission (46) – the presence of an increased number of shed cells at the lumen was apparent although without clear signs of damage to the epithelial lining. Such observations were consistent with previous reports showing rapid recovery of epithelial integrity following extensive exfoliation caused by N-9 exposure, both in mice (47) and pig-tailed monkeys (48). Histological analysis of middle colon tissues was also performed but no signs of toxicity were found in all treatment groups (**Figure 4.6**).

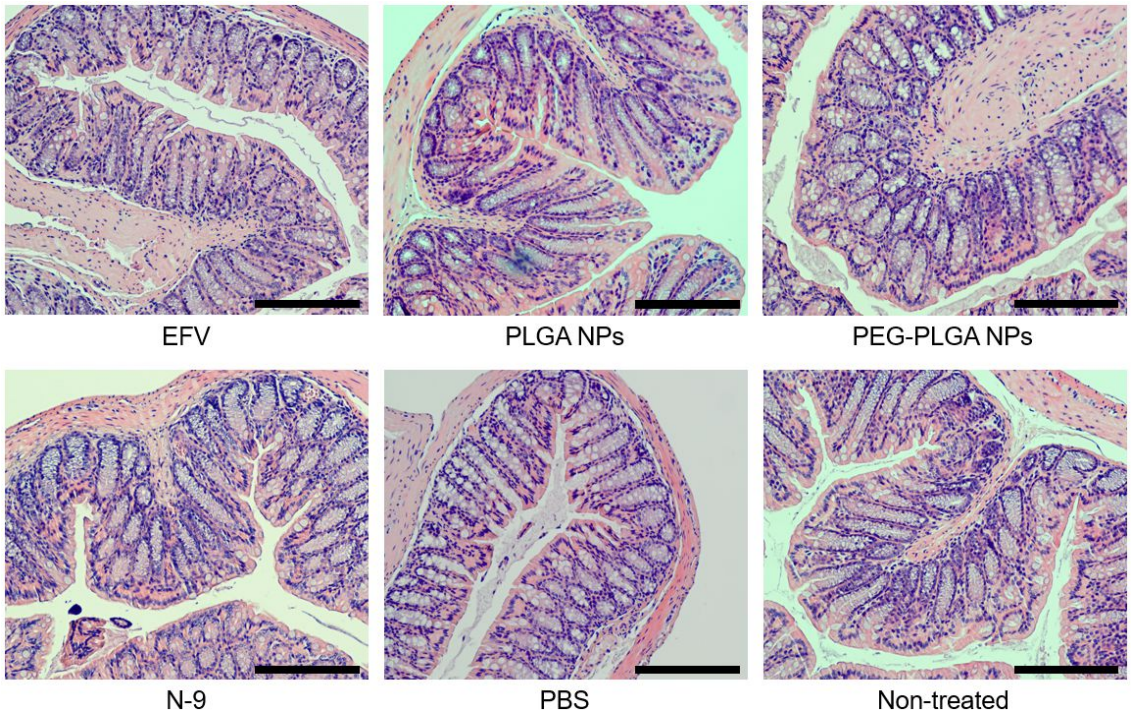

Figure 4.6. Representative H&E microphotographs of middle colon sections of mice treated with either free drug, EFV-loaded PLGA NPs or EFV-loaded PEG-PLGA NPs. Control groups included animals administered with N-9 or PBS, as well as mice undergoing no treatment (scale bar=200 μm).

Additional assay of IL-1β, IL-6, IFN-γ and TNF-α levels was performed in selected rectal lavage samples collected throughout the duration of safety studies.

Changes in cytokine/chemokine profiles have been shown valuable in assessing the onset of pro-inflammatory response (49, 50), which could potentially lead to an increased susceptibility to HIV transmission (51). Obtained results are presented in **Figure 4.7**. No significant differences were observed at any time point between baseline and 14 days thus suggesting that neither type of EFV-loaded NPs nor the free drug were able to trigger a pro-inflammatory response. Again, mild changes were only noted for N-9 at day 2 (IL-1β) and day 7 (IL-6 and TNF-α) as compared to PBS-treated mice. As in the case of histological analysis, rapid luminal clearance and epithelial turnover may mitigate larger imbalance in cytokine/chemokine levels. Together with increased cell shedding observed during histological analysis, data seem to confirm the harmful potential of N-9 and the usefulness of the mouse model in screening microbicide safety.

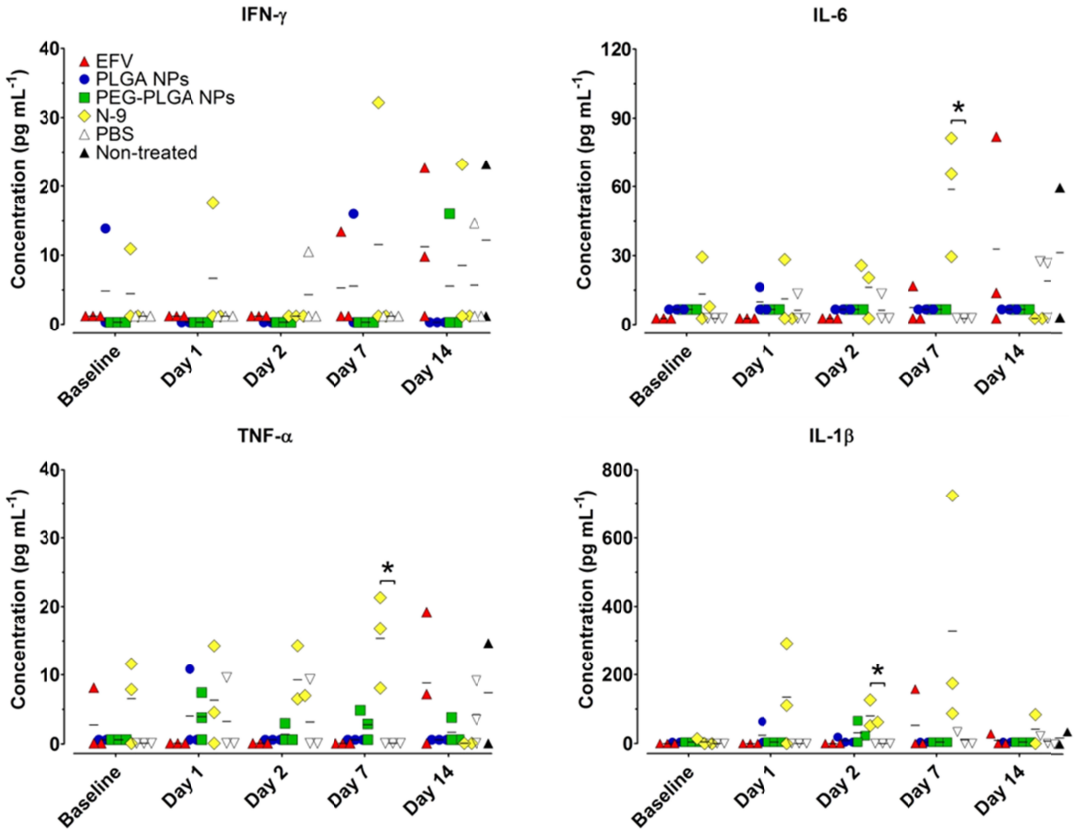

Figure 4.7. Levels of IFN-γ, IL-6, TNF-α and IL-1β in rectal lavage samples collected at 30 min before a new administration over 14 days. Individual values are presented for each cytokine/chemokine (horizontal bars stand for means; *n*=2-3). Note the different scales in *y*-axes. (*) denotes significant difference ($p<0.05$) when compared with PBS-treated animals.

4.5. Conclusions

Rectal microbicides hold the potential to play a key role in preventing sexual HIV transmission. Nanotechnology-based solutions offer new possibilities for developing safer and more effective products but work in the field remains scarce. In this work, we proposed a simple nanocarrier comprising FDA/European Medicines Agency (EMA)-approved materials for the delivery of the ARV drug EFV. In particular, we studied the effect of dense surface modification of EFV-loaded PLGA NPs with PEG (by means of poloxamer 407 adsorption) on local PK using a murine model. Both types of NPs were able to improve the colorectal availability of EFV after rectal administration as compared to the free drug. However, PEG modification of EFV-loaded NPs provided prolonged drug residence at higher levels at the lower colon, which could potentially translate into enhanced protection from HIV transmission. Our results seem to support the usefulness of mucus-diffusive nanocarriers in engineering effective and safe rectal microbicides. Nevertheless, future work is deemed necessary, namely regarding the correlation between enhanced local PK and protection from infection, as well as the design of dosage forms that could incorporate and further enhance the performance of PEG-PLGA NPs.

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CHAPTER 5

Conclusions and Future perspectives

5.1. Conclusions

Despite all the tremendous achievements in the field of HIV prevention, much work remains to be done. Microbicides, in particular those for rectal use, represent a promising strategy for preventing viral transmission. However, the development of effective, safe and acceptable products has been slow and characterized by a series of breakthroughs and setbacks. Product design and formulation are critical steps in the development process of rectal microbicides and nanotechnology-based approaches may just be the solution to bypass at least some of the shortcomings seen for to more conventional rectal dosage forms. Overall, rectal microbicide products should be shown able to effectively protect from HIV infection when used consistently, without raising any safety issues.

In order to comply with the overall aim and specific objectives established at the beginning of this work, PLGA NPs were developed using a nanoprecipitation method and poloxamer 407 – a triblock copolymer of PEG-PPG-PEG – was successfully used to establish a dense non-covalent PEG corona, thus rendering mucus diffusive properties to otherwise mucoadhesive PLGA NPs. PLGA NPs and PEG-PLGA NPs presented mean hydrodynamic diameter values around 200-220 nm, narrow size distribution and ZP values varying from slightly negative for PLGA NPs to near neutral for PEG-modified ones. In addition to ZP, different analytical techniques including ^{1}H NMR, EDS and contact angle measurements were able to be used in order to confirm the presence of poloxamer 407 chains at the surface of PEG-PLGA NPs, suggesting that dense coverage with PEG was in fact achieved. This corona was shown to be relatively resistant to desorption upon up to 24 h of washing *in vitro*. When administered intrarectally to mice, fluorescently-labeled PLGA NPs and PEG-PLGA NPs featured distinguishable behavior, as assessed by qualitative and quantitative techniques. Although both types of NPs were able to rapidly migrate and distribute throughout the last two thirds of the colon, only small fractions of the total administered NPs (up to around 15%) were able to be recovered from the colorectal lumen and tissues after 15 min post-administration. Retention of PEG-PLGA NPs was, however, consistently higher up to at least 2 h as compared to PLGA NPs (approximately 5- to 10-fold). Also, fluorescent microscopy imaging of transverse sections of the colon showed that PEG-PLGA NPs were able to better

spread over the epithelial surface and reach the deep folding of the large intestine, whereas distribution of PLGA NPs was mostly confined to the gut lumen. Moreover, PEG-PLGA NPs were demonstrated as being able to actually penetrate colorectal tissue.

The previous results encouraged moving forward with the comparison between PLGA NPs and PEG-PLGA NPs, namely concerning their potential as carriers for the model microbicide drug EFV. Both type of drug-loaded NPs were produced by easily adapting the developed nanoprecipitation method and were shown to maintain similar characteristics to non-loaded counterparts. High and comparable AE (>80%) and DL (around 4%) values were obtained for both PLGA NPs and PEO-PLGA NPs, which featured rapid drug release under sink condition. Drug association to NPs did not substantially affect the ARV activity of EFV against HIV-1 as assessed *in vitro*. However, an improvement to the intrinsic toxicity profile of the drug was noted, which led to larger SI values for drug-loaded NPs. Both types of NPs were also found to present low toxicity to different colorectal epithelial cells lines, thus showing to be potentially safe. Furthermore, NPs were able to be readily taken up by Caco-2 and HT29-MTX cells, while permeability studies using cell monolayer models showed a mild decrease in the transmembrane transport of EFV when associated to NPs. These results appear to emphasize the ability of NPs to interact with colorectal cells, which could be seen as beneficial in order to enable the drug to reach deeper mucosal levels and to be delivered to the vicinities of (or even directly to) HIV target cells, while potentially limiting systemic exposure.

The results described above considered as positive and justified the *in vivo* assessment of the PK and safety profiles of both types of EFV-loaded NPs. After rectal administration to mice, both types of nanosystems allowed reaching higher drug levels in colorectal tissues and fluids as compared to the free drug. Still, rapid depletion of EFV was observed, likely related to the typically fast leakage of materials observed in mice following rectal administration. Nonetheless and in line with data for *in vivo* distribution and retention of NPs, EFV appeared to have distributed retrogradely and reached several centimeters upwards into the colon. Generally, PEG-PLGA NPs displayed higher local drug levels as compared to PLGA NPs, although differences were only significant when considering colorectal tissues. Again, these results seemed to be in accordance with the distribution and retention patterns

observed for PEG-modified and non-modified PLGA NPs, and seem to support mucus diffusive NPs as better suited carriers for enhancing the PK of EFV at the colorectum. Systemic exposure to EFV was generally low when either free drug or drug-loaded NPs were used. Finally, both types of NPs were found safe after daily rectal administration for 14 days, thus indicating that particularly EFV-loaded PEG-PLGA NPs may be useful for developing a rectal microbicide product.

Overall, data presented in this work support that polymeric NPs may constitute interesting drug carriers for developing rectal anti-HIV microbicides. Adequate engineering of the surface properties of PLGA NPs, namely by granting mucus diffusive properties through means of dense, non-covalent coating with PEG, provides a simple yet powerful way to enhance microbicide drug levels at the colorectum. This, in turn, may allow conferring higher degree of protection against sexual transmission of HIV upon RAI.

5.2. Future perspectives

"Science never solves a problem without creating ten more."
George Bernard Shaw

Scientific research is a never ending story, typically raising other issues that require further assessment. The present work is no exception, presenting limitations and several aspects that may be improved and/or implemented in future studies. Some of the limitations of the present work were already discussed throughout the document and will not be again addressed in this section. However, some reflections on subsequent work and new directions seem appropriate.

Firstly, some aspects concerning the formulation of NPs could be re-visited. For example, other ARV drugs could be tested in order to confirm the broad potential of the proposed systems to deliver different molecules of interest. This would also allow developing combination microbicides based on nanocarriers featuring two or more ARV drugs, preferentially with different targets in HIV infection cascade. Co-association to NPs could be tested but individual incorporation of ARV drugs of interest during the production process would likely be an easier in order to fine tune

the properties of obtained nanosystems. The rapid *in vitro* release of EFV (and possibly of other candidate drugs) observed under forced sink conditions may be an issue requiring revision. Although PK results indicate that such behavior may not have occurred under physiological conditions, improvements could be pursued, namely by changing the composition of NPs (e.g., use of other types of PLGA, inclusion of complexing agents, etc.). Concerning the possibility for further functionalization, proposed NPs could benefit of the surface immobilization of specific targeting agents that could benefit the specific delivery of EFV to HIV-susceptible cells. The development of targeted nanosystems may help improving the efficacy and safety of microbicide products. Obvious targeting moieties include, among others, agents that are able to bind host cell surface receptors involved in HIV transmission and infection (*e.g.*, CD4, CCR5, DC-SIGN) (1). Another aspect that was not explored in this work concerns the development of actual products that could be able to be directly translated into clinical tested. NPs were used as a PBS suspension, which may be generally considered as representative of enemas. However, the influence of including additional excipients of interest (*e.g.*, preservatives, humectants, stabilizers, etc.) or even considering other dosage forms (*e.g.*, gels, soft capsules) on the performance of both types of NPs deserves further investigation. Other relevant issues pertaining to formulation development and clinical translation that should be further addressed even at this early stage of the development process include scaling-up, manufacturing, quality control and stability of drug-loaded NPs.

In addition to the already generated *in vivo* distribution and retention data, knowledge on the behavior of both PEG-modified and non-modified PLGA-based NPs in animal models that could proxy the reality of RAI would be helpful. For example, it may be expected that the presence of semen (or semen simulant) or the occurrence of simulated penile penetration can impact to some extent the distribution and retention of NPs in the colorectal compartment.

The *in vitro* ARV studies conducted were limited particularly regarding the type of infection model and number of HIV strains used. While TZM-bl cell-models are useful for early activity screening of ARV compounds, they lack the ability to mimic important features of *in vivo* scenarios typical of RAI such as the presence of mucosal fluids and semen, shear caused by penile penetration or resting bowel movement. Also, the absence of a complex mucosal tissue composition and

architecture limit the usefulness of TZM-bl cell-models. For example it would be interesting to use human colorectal explants in order to assess the ability of drug-loaded NPs to abbreviate infection by HIV. The inclusion of semen and/or native mucus, simulation of the natural depletion of compounds/carriers or testing of multiple viral exposures in such models is also possible and could provide interesting hints as to the potential of proposed NPs (2, 3). For instance, the role of semen in HIV infection by the rectal route is an important topic that remains poorly understood. While some data suggests the impairment of HIV infection by semen, another points that semen components may facilitate the HIV transmission (4). Moreover, some studies suggest that the influence of semen in HIV infections may depend on the antiviral agent used (5). So, it is important to understand the differences in the antiviral activity of EFV-loaded NPs in the presence of semen, since it may lead to variable results (6). Also, expanding the panel of HIV-1 strains used for testing ARV activity, namely by including resistant or MSM-derived transmitted/founder (T/F) viruses, would be interesting (7, 8).

Despite being useful, colorectal explants lack the definitive *in vivo* environment and the effect of some factors are not able to be studied in this kind of *ex vivo* models. Therefore, testing EFV-loaded NPs in animal models of infection would be of paramount interest. For, example, humanized murine models of HIV infection have been used to evaluate the effectiveness of vaginal and rectal microbicides (7, 9). Importantly, the relevance of PK data generated in the present study could provide an important starting point for planning experiments in humanized mice. Other relevant animal models for testing rectal microbicides include NHPs (10, 11). Despite important limitations, these are considered the most relevant species before translation into humans and, if possible, drug-loaded NPs should be tested in such models. Overall, the use of an *in vivo* efficacy model would provide a more realistic environment in order to understand the real impact of developed drug-loaded PLGA NPs in the prevention of HIV transmission (7).

Although generated data points out to the potential safety of developed nanosystems, such studies should be expanded in order to further support translation into clinical testing. For example, it would be interesting to study the toxicity of nanosystems in human colorectal explants, similar to those used for testing efficacy (12). Also, detailed and prolonged *in vivo* testing of the drug-loaded NPs, namely in

different animal models (*e.g.*, rats, rabbits) would be advisable. The local microbiota is essential to maintain the homeostasis in a symbiotic relationship with the host (13, 14). Any factor affecting microbiota may cause of profound alterations in this relationship (15). Furthermore, microbiota can have impact on the metabolism of various drugs and this possibility should be considered for EFV and EFV-loaded NPs. So, the evaluation of the interactions of NPs with rectal microbiota would be a relevant upgrade to the already conducted work.

5.3. References

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Biographical Note

Rute Nunes was born on May 29, 1985 in Campanhã, Porto, Portugal. She obtained her Bachelor of Science degree in Nursing in 2007 from Escola Superior de Enfermagem de Viana do Castelo, Viana do Castelo, Portugal. Further, she obtained her Master in Science degree in Pharmaceutical Sciences, granted by Instituto Superior de Ciências da Saúde do Norte (ISCS-N), Gandra, Portugal. She has been developing her work at Instituto de Investigação e Inovação em Saúde (I3S)/Instituto de Engenharia Biomédica (INEB), Porto, Portugal. Her research mainly focused on the rectal drug delivery of drugs, nanotechnology-based nanocarriers and rectal microbicides for the prevention of HIV transmission. She obtained several skills in the development and characterization nanosystems. She also has a broad training in cell culture techniques and cell-based assays as well as conduction of *in vivo* experiments in mice. Her research work resulted in two peer-reviewed original articles (one published and another submitted for publication), one review article and three book chapters as first author. Also, she actively collaborated in other projects that resulted in peer reviewed publications.